ANTIBACTERIAL ACTIVITY OF *Chrysanthemum cinerariaefolium* (PYRETHRUM) SECONDARY METABOLITES AND GREEN SYNTHESIZED SILVER NANOPARTICLES

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been presented in this university or any other for the award of a degree

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DEDICATION

I dedicate this work to my parents Mr and Mrs John Chemaoi and my daughters, Marvel and Mylla.

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ABSTRACT

The development of resistance to antibacterial agents by bacteria, drive efforts in bio prospecting for new novel compounds that can be used to target these resistant microorganisms. Plants are among natural sources of novel compounds with medicinal importance due to their desirable potency. Besides, plant phytochemicals can reduce metal ions to metal nanoparticles hence play important role in the green synthesis of nanoparticles. In the current study, bioactive compounds against selected bacteria were isolated from pyrethrum plant *Chrysanthemum cinerariaefolium* by carrying out bioassay-guided fractionation. The isolated compounds were characterized using 1D and 2D Nuclear Magnetic Resonance (NMR). Extracts of organic solvents and aqueous of *C. cinerariaefolium* were also used in the green synthesis of silver nanoparticles (Ag NPs) via reduction of silver ions present in silver nitrate. Synthesis involved mixing a fixed ratio of plant crude extracts with silver ions and storing the mixture in the dark. Observation of a color change to brown signified the formation of the nanoparticles. The nanoparticles were characterized using UV-Vis, Scanning Electron Microscopy, Transmission Electron Microscopy, EDX (energy dispersive X-ray analysis), and Fourier-Transform Infrared Spectroscopy (FTIR). The compounds isolated were (Z)-2 methyl-4-oxo-3-(pent-2-en-1-yl)cyclopent-2-en-1-yl2,2-dimethyl-3-(2-methylprop-1-en-1-yl) cyclopropane-1-carboxylate (jasmolin I), 2-methyl-4-oxo-3(Z)-penta-2,4-diene-1-yl)cyclopent-2-en-1-yl3-(E)-3-methoxy-2-methyl-3-oxoprop-1-en-yl-2,2-dimethylcyclopropane-1-carboxylate (pyrethrin II), and (Z)-2-(but-2-en-1-yl)-4-hydroxy-3-methylcyclopent-2-en-1-one (cinerolone). The compounds showed more activity on the bacteria as a mixture in the ratio of 1:1:1 than individual compounds, with MIC of 25 mg/ml against *Pseudomonas aeruginosa*. The compounds can therefore be used as lead compounds in drug discovery against bacteria. All the nanoparticles formed were generally spherical in shape. The smallest and largest nanoparticles had sizes of 22.8± 17.5 nm and 75.3± 19.7 nm and they belonged to dichloromethane-Ag NPs and ethyl acetate-Ag NPs respectively. The particles exhibited size-dependent activity on the selected bacteria. Safety studies on the nanoparticles and pyrethrum extracts on Vero cells showed that they were not cytotoxic hence safe for utilization in drug discovery. Pyrethrum plant therefore possesses phytochemicals that can be used in green synthesis nanoparticles. Other plants should be exploited to ascertain their ability to synthesis nanoparticles.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
ASTM	American Society for Testing and Materials.
ATCC	American Type Culture Collection
CDC	Center for Disease Control and Prevention
COSY	Correlation Spectroscopy
DEPT	Distortionless Enhancement by Polarization transfer
DMSO	Dimethyl sulphoxide
EDS/EDX	Energy Dispersive X-Ray Analysis
EO	Essential Oil
FTIR	Fourier Transform Infrared Spectroscopy
HaCaT	Cultured Human Keratinocyte
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear single quantum correlation experiment
IC_{50}	Half-maximal inhibitory concentration
KALRO	Kenya Agricultural and Livestock Research Organization
MBC	Minimum Bactericidal Concentration
MIC	Minimum inhibitory concentration
MCF	Malignant catarrhal fever
MDR	Multi-drug-resistant
MEM	Minimum Essential Media
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MSSA	Methicillin-Susceptible *Staphylococcus aureus*
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NCCLS	National Committee for Clinical Laboratory Standards
NCI	National Cancer Institute
NLRP	Nucleotide-binding oligomerization domain
NMR	Nuclear Magnetic Resonance
NPs	Nanoparticles
OD	Optical Density

PBK	Pyrethrum Board of Kenya
PBP	*Penicillin*-binding proteins
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
PMAA	Poly (methacrylic acid)
PVP	Polyvinylpyrrolidone
ROS	Reactive oxygen species
SEM	Scanning Electron Microscopy
SPR	Surface plasmon resonance
TEM	Transmission Electron Microscopy
TLC	Thin Layer Chromatography
TLR	Toll-like receptors
TMS	Tetramethylsilane
UNAIDS	United Nations Programme on HIV and AIDS
UNICEF	United Nations International Children's Emergency Fund
UV-VIS	Ultraviolet-visible Spectroscopy
VLC	Vacuum Liquid Chromatography
VRE	*Vancomycin-Resistant Enterococci*
WHO	World Health Organization
XRD	X-Ray Diffraction

CHAPTER ONE

INTRODUCTION

1.1 Background information

Microbial infections continue to be a growing concern in the world due to resistance to current antimicrobial agents. According to estimates by the Center for Disease Control and Prevention (CDC), approximately 2.8 million antibiotic-resistant infections occur in the U.S yearly with about 35,000 deaths (CDC, 2019). In sub-Saharan Africa, 2.6 million babies required treatment for severe bacterial infection in the first month of life in 2012 (Anna *et al.,* 2014). In a study done in South China, bacteria were the leading causative agent of food-borne illness with 44.93% followed by poisonous plants at 33.33% (Li *et al.,* 2018). Bacteria are the oldest form of life on earth. They are tiny hence referred to as micro-organisms. They are abundant since they exist almost everywhere on earth i.e in air, water, soil, rocks, plants, animals, and the human body, and extreme conditions such as hot springs and acidic environments (Fredrickson *et al.,* 2004). Among the beneficial effects of bacteria is in biotechnology where it is used for breakdown of oil spills, fermentation of cheese and yogurt, recovery of metals in the mining sector, and the manufacture of antibiotics (Ishige *et al.,* 2005).

Among the harmful effects of bacteria is causing a variety of bacterial infections affecting humans, animals, and plants (Reta *et al.*, 2019). Antibacterial agents currently used to treat bacterial infections work by inhibiting the growth of micro-organisms or kill them by interfering with cell wall synthesis and DNA replication (Senka *et al.,* 2008). Regrettably, overuse and misuse of these antibacterial agents have led to the development of resistance by bacteria thus rendering these agents ineffective in the treatment of infections associated with these micro-organisms (Davis & Davis, 2010). The concerns about the development of resistance to antimicrobial agents by bacteria drive efforts in bioprospecting for new novel compounds and formulations that can be used to target these resistant microorganisms. Plants are among natural sources of novel compounds with medicinal importance due to their desirable potency and low toxicity (Stamets, 2002). As a result, most antimicrobial agents that have been used against bacteria have been derived from natural products such as plants (Stamets, 2002). Other than acting as a source of novel compounds, plants also are a source of phytochemicals that can reduce metal ions to metal nanoparticles hence playing roles in the green synthesis of nanoparticles. The notable nanoparticles majorly synthesized by plant phytochemicals are gold and silver nanoparticles (Vo *et al.*, 2019).

In the current study, pyrethrum extracts were subjected to bioassay-guided fractionation inorder to isolate novel compounds that possess antibacterial activity against *Pseudomonas aeruginosa, Shigella sonnei, Methicilin Resistant Staphylococcus areus (MRSA), and Staphylococcus aureus.* Besides isolation of antibacterial compounds, pyrethrum extracts were also used in the green synthesis of silver nanoparticles. This follows the fact that silver ions are medically known for their antibacterial properties (Devi & Joshi, 2015). The pyrethrum plant was chosen because of its bioefficacy. The plant has a long history of use as an insecticide due to the production of pyrethrins that have been noted to possess insecticidal properties (Ireri *et al.,* 2011). In the past decades, the production of the crop increased steadily due to the demand for natural pyrethrins for insecticide production. During the1980s and 1990s, Kenya was a global leader in pyrethrum production, contributing over 70% to the global market (Grdiša *et al.,* 2009). The sub-sector supported more than 200,000 small-scale growers, 3,000 workers directly employed by the pyrethrum board of Kenya (PBK), and over 2 million people deriving their livelihood from the industry either directly or indirectly. The sub-sector was a major foreign exchange contributor with earnings rising to KSh 2.1 billion in 1996 (Kariuki, 2013).

A decline has been seen in the production of pyrethrum globally in recent years, with Kenya being affected. This is attributed to the introduction of low-cost synthetic pesticides known as pyrethroids (Grdiša *et al.,* 2009). These pyrethroids are environmentally unfriendly, develop resistance quickly, and are toxic (Thatheyu & Selvam, 2013). The decline in the pyrethrum industry in Kenya as a result of pyrethroids has been drastic, with export declining from the initial 18,000 tonnes in 1980-1982 to as low as less than 10 tonnes per year (Mureithi, 2011). Apart from having insecticidal properties, plants from the genus *Chrysanthemum* have been reported to possess other medicinal importance (Jung, 2009). For example flowers of *Chrysanthemum morifolium* Ramat and its herbal infusions are used in the treatment of bacterial, viral infections, sinusitis, blood pressure, digestive problems, skin problems, influenza virus PR 3, leptospira, HIV-1, human colon cancer, headache, dizziness, sore throat, hypertension, flu, and cough (Yeasmin *et al.,* 2016).

Finding new biomedical uses of pyrethrum secondary metabolites will help in the fight against infectious diseases associated with bacteria. It will also increase the demand for the crop, as new uses of the plant shall be exploited. Pyrethrum grows in high altitude areas ranging from 1500-3000 meters above sea level and requires rich volcanic soils with good draining. The soil should also have a minimum pH of 5.4 to slightly alkaline and rich in nutrients (phosphorus, calcium, and magnesium). Besides, rainfall should be between 762-

1270 mm spread throughout the year with fertile and well-drained soils of moderate organic matter (Wandahwa *et al.*, 1996). In the present study, *C. cinerariaefolium* was therefore obtained from Elgeyo-Marakwet County, which is one of the pyrethrum growing ecological regions in Kenya.

1.2 Statement of the problem

Bacterial infections continue to be a growing concern in the world. These infections are aggravated by the rapid development of resistance to current antibacterial agents, due to misuse of various antibacterial drugs. This therefore, has necessitated a search for novel compounds that can be used as lead compounds in drug discovery hence aid in combatting these infections. Due to their small sizes, silver nanoparticles possess novel physicochemical and biological properties such as enhanced reactivity and the ability to cross-cell and tissue barriers. They have also been reported to trap silver ions, which have a high affinity for sulphur containing proteins and phosphate containing groups such as the DNA. They also generate reactive oxygen species, which damage bacterial cellular components. As a result, silver nanoparticles have found widespread applications in the medical field, cosmetics and industrial sectors. Common methods of synthesizing the metallic nanoparticles are chemical and physical. However, these methods are expensive and use toxic chemicals.

1.3 Objectives

1.3.1 General objective

To study antibacterial activity of *Chrysanthemum cinerariaefolium* secondary metabolites and green synthesized silver nanoparticles.

1.3.2 Specific objectives

i. To determine the antibacterial activity of secondary metabolites isolated from *C. cinerariaefolium* against *P. aeruginosa, S. sonnei, MRSA, and S. aureus*.
ii. To characterize the isolated compounds from *C. Cinerariefolium* using NMR.
iii. To characterize synthesized silver nanoparticles using Transmission Electron Microscope (TEM), Scanning Electron Microscope (SEM), UV-vis, and Fourier-Transform Infrared Spectroscopy (FTIR).
iv. To determine the bioactivity of the synthesized silver nanoparticles against *P. aeruginosa, S.sonnei, MRSA,* and *S. aureus.*
v. To determine the cytotoxicity of the isolated compounds and the synthesized silver nanoparticles against Vero cells.

1.4 Hypotheses

i. There is no significant difference in the activity of isolated metabolites from *C. cinerariaefolium* against *P. aerugenosa, S. sonnei, MRSA,* and *S. aureus.*
ii. Metabolites from *C. cinerariefolium* have similar chemical characteristics.
iii. The synthesized silver nanoparticles have similar characteristics.
iv. There is no significant difference in the activity of the synthesized silver nanoparticles against *P. aeruginosa, S. sonnei, MRSA,* and *S. aureus.*
v. Isolated compounds and silver nanoparticles lack cytotoxicity activity against Vero cells.

1.5 Justification

Plants are among nature sources of novel compounds with medicinal importance. Determination of the antibacterial activity of isolated compounds from the pyrethrum will help in identifying lead compounds that can be used in the production of new antibacterial drugs. Nanotechnology is an emerging field that has opened new horizons in nanomedicine. The use of green synthesis in the production of silver nanoparticles is not only cheap but also safe. Formulation of silver ions to silver nanoparticles would therefore improve the already existing antibacterial activity present in silver. Identification of new biological uses of the pyrethrum plant besides its historical insecticidal use is important in increasing the demand for the crop as new uses will be exploited.

CHAPTER TWO
LITERATURE REVIEW

2.1 Overview of infectious diseases

Infectious diseases are among the top 10 causes of mortality and the leading cause of disability-adjusted life years globally (WHO, 2017). They are caused by bacteria, fungi, parasites, and viruses. Most of the developing nations are affected by the morbidity and mortality resulting from infectious diseases with infants and children being the most vulnerable group (UNICEF, 2004). In the year 2011, severe bacterial infections were reported to account directly for roughly one-third of neonatal deaths in sub-Saharan Africa, South Asia (SA), Latin America, and the Caribbean (Liu *et al.*, 2012). Viral infections are also a menace globally with approximately 36.7 million people globally infected with HIV in 2015 according to UNAIDS, (2016). Moreover, coronavirus disease (COVID-19) has been reported to infect 2.1 million people globally and cause 142,229 deaths within 4 months since its discovery (WHO, 2020).

There are many reasons for the emergence and re-emergence of infectious diseases globally. They include a breakdown of public health measures in the face of epidemic transitions, microbe adaptation, and ability to mutate, the transmission of several pathogens between animals and humans, poor sanitation, poverty, ignorance, and of great concern is the global emergence of resistance of infectious pathogens to many first-line drugs (WHO, 2017). According to the Center for Disease Control and Prevention (CDC), antibiotic resistance is responsible for around 2 million infections, more than twenty thousand deaths and, costs $55 billion each year in the United States (CDC, 2013). The chief infections associated with antimicrobial resistance to conventional drugs include multidrug-resistant *tuberculosis, methicillin-resistant Staphylococcus aureus,* and *Vancomycin-resistant enterococci* (Fair & Tor, 2014). Due to multidrug resistance, TB was reported to cause an estimated 1.8 million deaths in 2015, including 0.4 million deaths associated with HIV co-infection (WHO, 2016). These infectious diseases are therefore associated with a serious impact on public health globally. With these observations, proper mitigation that includes prevention and the development of new treatments is therefore needed.

2.2 Bacteria and common bacterial infections

Bacteria are single-celled microbes with simple cell structures. They have different shapes and sizes and are typically 0.5-5.0 micrometers in length with a few species such as *Thiomargarita namibiensis* and *Epulopiscium fishelsoni* being up to half a millimeter long (Schulz & Jorgensen, 2001). They do not have a membrane-bound nucleus, and their genetic material is a single circular DNA that is dispersed in the nucleoid region of the cytoplasm (Thanbichler *et al.*, 2005). They reproduce by binary fission and under optimal conditions; bacteria can grow and divide extremely rapidly with their populations doubling every 9.8 minutes (Koch, 2002). Bacteria are classified into gram-positive and gram-negative based on gram staining. Gram staining differentiates bacterial species based on chemical and physical properties of their cell walls peptidoglycan, which is present in a thick layer in gram-positive bacteria (Ryan & Ray, 2004). Bacteria are both harmful and beneficial.

Among the beneficial effects of bacteria is that they form part of gut and skin microflora in humans important for protection against pathogenic micro-organism (Sears, 2005). Some bacteria such as *Bacillus thuringiensis* have been used in biological pest control (Aronson & Shai, 2001). Bacteria have enabled scientists to determine the function of genes, enzymes, and metabolic pathways. This is due to their ability to quickly grow and the relative ease with which they can be manipulated (Serres *et al.*, 2001). Other uses include degradation of a variety of organic compounds such as those found in oil spills and their use in fermentation (Johnson & Lucey, 2006). The harmful effects of bacteria are due to their ability to cause infectious diseases, which are of public health concern. Gram-negative bacteria are a cause of more than 30% of hospital-acquired infections that predominate in cases of ventilator-associated pneumonia (47%) and urinary tract infections (45%) (Hidron *et al.*, 2008).

There is a range of gram-negative organisms responsible for nosocomial infections. *Enterobacteriaceae* family is the most commonly identified group and others are *P. aeruginosa*, *staphylococci*, and *Actinobacteria* (Gaynes & Edwards, 2005). Gram-positive bacteria include *methicillin-resistant Staphylococcus aureus (MRSA)*, *methicillin-susceptible Staphylococcus aureus (MSSA)*, and *vancomycin-resistant enterococci (VRE)* (Rivera & Boucher, 2011). In livestock, bacterial diseases cause *anthrax*, *Salmonellosis*, *Leptospirosis*, and *Mycobacterium* infections (Odontsetseg *et al.*, 2005). Bacterial infections cause a drastic decline in farm produce. In Nepal, bacterial stalk rot *Erwinia chrysanthemi* caused up to 80%

yield loss along with fungal diseases in maize in the plains (Burlakoti & Khatri-Chhetri, 2004).

2.3 General overview of pathogens used in this study

2.3.1 *Staphylococcus aureus*

These bacteria are commensal as well as pathogenic to humans. Being pathogenic, they cause bacteremia, infective endocarditis, skin and soft tissue infections, osteoarticular infections, epidural abscess, meningitis, toxic shock syndrome, urinary tract infections, and pleuropulmonary infections (Tong *et al.,* 2015). The anterior nares are the principal ecological niche where the bacteria inhabit in humans. The nasal carriage colonization by *S.aureus* escalates the risk of infection especially in hospital settings (Kluytmans & Wertheim, 2005).

2.3.2 *Methicillin-resistant Staphylococcus aureus*

These bacteria are those *S. aureus* strains carrying a *mecA* gene, which codes for an extra penicillin-binding protein, PBP2a. The presence of mecA makes *MRSA* resistant to nearly all beta-lactam antibiotics. The mechanism of action of beta-lactam antibiotics is the inactivation of penicillin-binding proteins (PBPs), which are key enzymes for bacterial cell wall synthesis. Nonetheless, the beta-lactams have only a low affinity towards PBP2a, thus this enzyme evades inactivation and continues to carry out their role of cell wall synthesis and survival of bacteria in presence of beta-lactam antibiotics (Fuda *et al.*, 2004).

Penicillin being the first beta-lactam antibiotic discovered in 1928 was found to be most effective against *S. aureus* infections. Unfortunately, soon after its introduction, in the year 1940, incidences of *S. aureus* resistance to penicillin were reported (Rammelkamp & Maxon, 1942). Their resistance to penicillin was due to the production of plasmid-encoded beta-lactamase enzyme (penicillinase) which enzymatically cleaved the beta-lactam ring of penicillin making the antibiotic inactive (Bondi & Dietz, 1945). In the 1950s, the resistance to penicillin by *S. aureus* was restricted to hospitals. By late the 1960s, more than 80% of *S. aureus* resistant isolates irrespective of community and hospital origin developed resistance to penicillin due to plasmid transfer of penicillinase gene (blaZ) and clonal dissemination of resistant strains (Lowy, 2003).

Following resistance to penicillin, methicillin semi-synthetic penicillin that withstood the enzymatic degradation of penicillinase was introduced in 1961. Nevertheless, in less than a year resistance of *S. aureus* to methicillin was reported (Jevons, 1961). In the next 10 years, a growing number of *MRSA* outbreaks were reported in different parts of the world especially from the European countries (Ayliffe, 1997). The prominent feature of the resistant reports was that the incidences were from hospitals hence *MRSA* emerged as a hospital-borne pathogen (Gnanamani *et al.*, 2017).

2.3.3 *Pseudomonas aeruginosa*

It is a gram-negative, aerobic rod-shaped bacterium. It is unable to ferment lactose and is oxidase-positive (CDC, 2014). It is found in diverse environments such as water, plants, soil, and on the epidermis of animals. In nature, it is usually found as plankton swimming through water or as a biofilm (CDC, 2014). It has a large genome coding for 5570 genes encoding an unusually high proportion of proteins involved in regulation, transport, and virulence functions with 0.3% of the total genes code for proteins involved in anti-microbial resistance. Moreover, the genome is greatly flexible, with 10% of genes organized in 'pathogenicity islands', encompassing variable genes coding for virulence factors and the ability to easily acquire large mobile genetic elements(integrons) encoding resistance genes (Kipnis *et al .*, 2006).

P. aeruginosa is one of the clinically and epidemiologically significant bacteria. It causes opportunistic infections in immunocompromised individuals and nosocomial infections (Pollack, 2000). In immunocompromised or patients that have experienced significant trauma, colonization of *P. aeruginosa* in the respiratory tract has been linked with sepsis and death with a mortality rate approximately at 50% for the said patients (Iglewski, 1996). Infections caused by *P. aeruginosa* associated with hospital settings include nosocomial threats for patients with ventilation machines, cancers, and burns. In neutropenic cancer patients undertaking chemotherapy, bacteraemia with *P. aeruginosa* is a common complication (Krcmery *et al.,* 2006). Bacteraemia and septicaemia can likewise occur in patients suffering from immunodeficiency related to AIDS, diabetes mellitus or severe burns (Sligl *et al.,* 2006). Approximately 12% of hospital-acquired urinary tract infections are also linked to *P. aeruginosa* (Obritsch *et al.*, 2005).

Several virulence factors enhance the pathogenicity of *P. aeruginosa*. These include factors that facilitate adhesion of the bacteria or disruption of host cell signaling pathways while targeting the extracellular matrix. Virulence factors acting in different ways to enhance the pathogenicity of the bacteria include lipopolysaccharide, flagellum, type IV Pili, type III secretion system, exotoxin A, proteases, alginate, quorum sensing, biofilm formation, type VI secretion systems, and oxidant generation in the airspace (Rocha *et al.,* 2019).

Lipopolysaccharide is important in the activation of the host's innate immune system factors that include (TLR4, NLRP1, NLRP2, and NLRP3) and adaptive immune responses, which eventually cause dysregulated inflammation responses that contribute to morbidity and mortality (Mandell *et al.,* 2005). Some proteases produced by *P. aeruginosa* such as metalloproteases of type elastase Las B destroy host tissue hence play a significant role in both acute lung infections and burned wound infections (Bielecki *et al.,* 2008).

2.3.4 *Shigella sonnie*

It is a rod-shaped, gram-negative facultative, intracellular, nonmotile, non-spore-forming, anaerobic pathogen that is differentiated from the closely related *E. coli* based on pathogenicity, physiology (failure to ferment lactose or decarboxylating lysine), and serology (Kelmani & Chidre, 2018). They are catalase-positive, oxidase, and lactose-negative. They ferment sugars, usually without forming gas. The organism thrives at temperatures ranging from 20^{o}C and 46^{o}C, with an optimum at 37^{o}C and a pH range of 5.0 to 7.5 (Ranjbar *et al.*, 2008).

S. sonnie causes shigellosis a global endemic characterized by abdominal pain, tenesmus, watery diarrhea, and/or dysentery (multiple scanty, bloody, mucoid stools), abdominal tenderness, fever, vomiting, dehydration, and convulsions (Kotloff *et al.*, 2013). The disease occurs due to invasion of the terminal ileum, the epithelium lining, rectum, and colon by *Shigella* species (Al-Dahmoshi *et al.,* 2020). Yearly, there are 165 million cases of shigellosis worldwide with 99% of mortality and morbidity occurring in Africa. Out of 1.1 million deaths reported in Africa annually, 69% are in children aged less than five years (Kotloff *et al.*, 1999). The high occurrences of *Shigella* in the developing countries are mainly attributed to the absence of clean water, poor hygiene, malnutrition, and person-to-person transmission in crowded or unhygienic environments such as informal settlements and prisons (Torres, 2004).

They contain changeable or removable elements that exist in a diversity of groups depending on the species, and subtypes of *Shigella* (Yang *et al.,* 2005). Moreover, *Shigella* can down-regulate the production of antimicrobial peptides involving human β-defensin hBD-3 and CCL20. It also induces defective dendritic cell recruitment hence allowing increased replications, vigorous infection of contiguous cells, and downregulation of immunologic response (Kobayashi *et al.,* 2013).

2.4 Antibacterial agents

2.4.1 Antibiotics

Antibiotics were considered ground-breaking discoveries in the field of medicine, in the 20th century since morbidity and mortality from infectious diseases drastically reduced (Sascha *et al.*, 2007). These antibiotics work by killing the bacteria (bacteriocidal) or inhibit the growth of these organisms (bacteriostatic). Most of the bactericidal agents inhibit DNA, RNA, and cell wall synthesis while bacteriostatic agents inhibit protein synthesis that is essential for bacterial growth (Pankey & Sabath, 2004). Common classes of antibiotics classified based on chemical or molecular structures include beta-lactams, macrolides, tetracyclines, quinolones, aminoglycosides, sulphonamides, glycopeptides, and oxazolidinones (Adzitey, 2015; Frank & Tacconelli, 2012).

a) β-lactams

These are antibiotics that have the β-lactam ring and include penicillins, cephalosporins, carbapenems, and monocyclic β-lactams (King *et al.*, 2016). They inhibit transpeptidation of the peptidoglycan layer, hence disrupting the bacterial cell wall leading to lysis (Bush & Bradford, 2016). Both gram-negative and gram-positive bacteria have proteins involved in the transpeptidation and penicillin-binding proteins (PBPs) (Bush & Bradford, 2016). All penicillins have a basic structure that consists of a beta-lactam ring, a thiazolidine ring, and a side chain (6-aminopenicillin acid) as shown in figure 1.

Beta-lactam ring determines the antibacterial activity of the penicillins. Penicillins are active against both gram-negative and gram-positive bacteria and are categorized depending on their structure and activity (Wright, 1999). Some penicillins such as amoxicillin and piperacillin may be combined with a β-lactamase inhibitor to exert activity against β-lactamase-producing such as *Enterobacteriaceae* spp and *P. aeruginosa* (Perry & Markham, 1999). They are used to treat bacterial infections arising from penicillinase-producing, *Methicillin-susceptible Staphylococci* and *Streptococci Proteus mirabilis,* some *Escherichia coli, Klebsiella pneumonia*, *Haemophilus influenza*, *Enterobacter aerogenes*, and some *Neisseria* (Pegler & Healy, 2007).

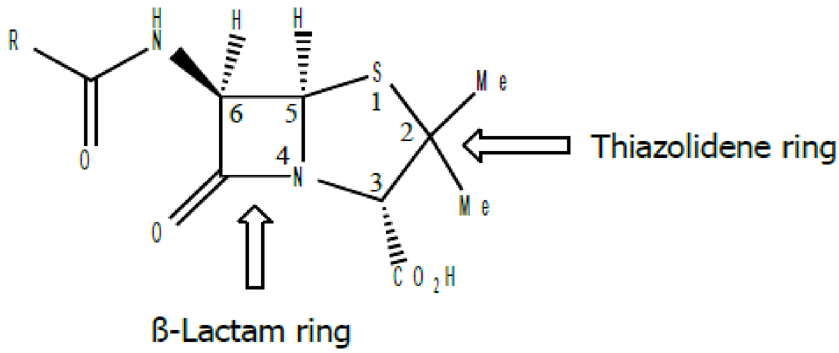

Figure 1: Basic structure of penicillin. It has a beta-lactam ring, a thiazolidine ring, and a side chain (Holten & Onusko, 2000).

Cephalosporins are divided into generations (1st-5th) following their target organism. Later versions of cephalosporins are more effective against gram-negative pathogens whereas lower-generation cephalosporins possess activity more against gram-positive bacteria. Cefepime (4th generation) is an exception drug with gram-positive activity similar to first-generation and gram-negative activity equivalent to third-generation cephalosporins (Harrison & Bratcher, 2008). They have a β-lactam ring like the penicillins but instead of a thiazolidine ring, cephalosporins have a 6-membered dihydrothiazine ring. Figure 2 shows the basic structure of cephalosporin.

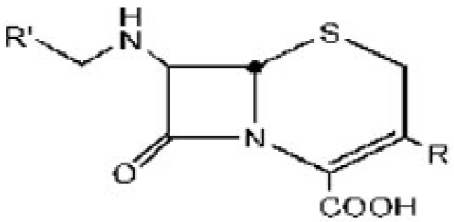

Figure 2: Cephalosporin basic structure. Instead of a thiazolidine ring, cephalosporins have a 6-membered dihydrothiazine ring (Pegler & Healy, 2007).

Carbapenems are beta-lactams, famous for their ability to resist the hydrolytic action of the beta-lactamase enzyme. They possess the broadest spectrum of bioactivity and potency against gram-positive and gram-negative bacteria. Due to their bioefficacy, they are frequently referred to as “antibiotics of last resort.” Patients who are severely ill or suspected of harboring resistant bacteria are usually given carbapenems (Meletis, 2016).

b) Fluoroquinolones

They include ciprofloxacin, levofloxacin, and moxifloxacin (Aminov, 2016). They work by blocking bacterial chromosome replication through inhibition of bacterial DNA gyrase (an enzyme responsible for the supercoiling of chromosomal DNA in bacteria for efficient cell division) (Hiasa & Shea, 2000). They are active against both gram-negative and gram-positive bacteria. Bacterial-resistant genes against fluoroquinolones include gyrA, gyrB, parC, and qnr. They act by reducing the formation of Gyrquinolone-DNA complexes (Aminov, 2017). Fluoroquinolones are frequently used as a first-hand treatment of febrile upper urinary tract infections and outpatient management of *P. aeruginosa* infections (Rotschafer *et al.,* 2011). They are also used in the combination treatment of multi-drug resistant *M. tuberculosis* (Caminero *et al.,* 2013) and prosthetic joint infections (Berdal *et al.,* 2005). All fluoroquinolones have fluorine at position 6 as shown in figure 3.

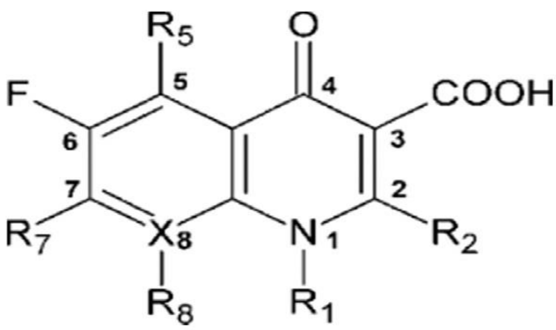

Figure 3: Floroquinone phamacore with fluorine molecule at position 6 (Brar *et al.*, 2020).

c) Macrolides

In this group, the first antibiotic was discovered and isolated in 1952 as a metabolic product of a soil-inhabiting fungus *Saccharopolyspora erythraea* formerly known as *Streptomyces erythraeus* (Moore, 2015). Their structure has unsual deoxy sugars i.e L-cladinose and D-desosamine attached to 14-, 15-, or 16-membered macrocyclic lactone rings. Those with 14-membered macrocyclic lactone rings include erythromycin, clarithromycin, and dirithromycin whereas the 16-membered ring structure is found in spiromycin, midecamycin, and micamycin. Azithromycin is a 15- membered ring structure as shown in figure 4 (Kwiatkowska *et al.,* 2013). They are usually administered to patients that are allergic to penicillin since they have an enhanced spectrum of activity than penicillins (Moore, 2015). They work by inhibiting protein synthesis. This is by attaching to bacterial ribosomes leading to the prevention of incorporation of amino acid to polypeptide chains during protein synthesis (Etebu & Arikekpar, 2016).

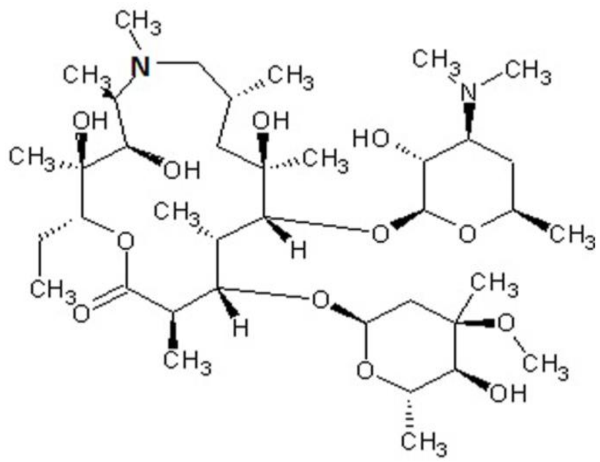

Figure 4: Basic structure of azithromycin with unusual deox sugars (Imperi *et al*., 2014).

d) Tetracyclines

The first member of this class is chlortetracycline (Aureomycin) discovered in 1945 from a soil bacterium of the genus *Streptomyces* (Sanchez *et al.,* 2004). Their structure has four (4) hydrocarbon rings as shown in figure 5. Their members are grouped into different generations depending on the method of synthesis. First-generation include those obtained by biosynthesis with members such as tetracycline, chlortetracycline, oxytetracycline, and demeclocycline. Second-generation members are derivatives of semi-synthesis and they include doxycycline, lymecycline, meclocycline, methacycline minocycline, and rolitetracycline. Third generations are obtained from total synthesis such as tigecycline (Fuoco, 2012). For enhanced absorption, patients are usually advised to take tetracyclines at least two hours before or after meals. Besides treating bacterial infections, tetracyclines have proven to be used to treat other infections such as malaria, elephantiasis, amoebic parasites, and rickettsia (Sanchez *et al.*, 2004).

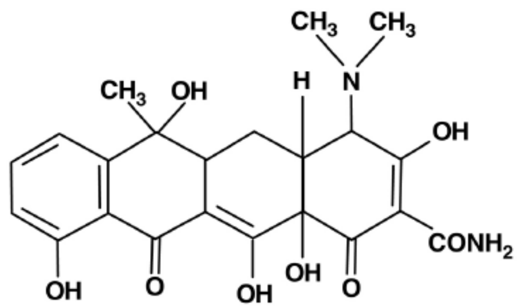

Figure 5: Structure of tetracyclines. The structure has four (4) hydrocarbon rings (Chopra & Roberts, 2001).

e) Oxazolidinones

These are the recent synthetic drugs to be approved for use in the year 2000 with linezolid (figure 6) being the first member. The mode of action is not yet fully understood though it has been reported to interfere with protein synthesis. This is by binding to the P site of the ribosomal 50s subunit (Bozdogan & Appelbaum, 2004). They have a broad spectrum of activity against resistant strains of bacteria that include *methicillin* and *vancomycin-resistant staphylococci*, *vancomycin-resistant enterococci*, *penicillin-resistant pneumococci*, and anaerobes (Bozdogan & Appelbaum, 2004). The common diseases treated by linezolid are respiratory tract and skin infections caused by gram-positive bacterial pathogens (Moellering, 2003). This drug is used restrictively due to its severe side effects associated with it, which include bone marrow suppression, polyneuropathy, anemia, and thrombocytopenia (Eckmann & Dryden, 2010; Kuter & Tillotson, 2001).

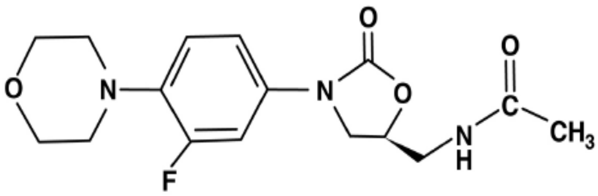

Figure 6: Linezolid (Oxazolidinones) structure (Leach *et al*., 2007).

f) Sulphonamides

They are the first group of antibiotics reportedly used in medicine and veterinary and they include sulfamethoxazole (Eyssen *et al.*, 1971). They inhibit both gram-positive and gram-negative bacteria such as *Nocardia*, *E.coli*, *Klebsiella*, *Salmonella*, *Shigella*, and *Enterobacter*. Other infections treated using sulphonamides are tonsillitis, septicemia, meningococcal meningitis, bacillary dysentery, and some urinary tract infections (Eyssen *et al.*, 1971). Studies have shown that sulphonamides likewise inhibit cancerous cells (Xu *et al.*, 2014). Although sulphonamides are efficient in treating various infections, they should be cautiously used due to the side effects associated with them. The side effects include urinary tract disorders, haemolytic anemia, porphyria, and hypersensitivity reactions (Choquet-Kastylevsky *et al.*, 2002; Slatore & Tilles, 2004;). Most sulphonamides are antibacterial possess aromatic amine as shown in figure 7.

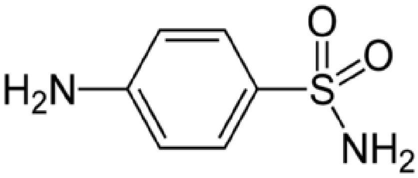

Figure 7: Structure of sulphonamides (Yousef *et al.*, 2018).

g) Glycopeptides

They were originally obtained as a natural product. However, semi-synthetic derivatives have been made in the last 20 years, which have greatly improved pharmacokinetic properties (Kahne *et al.*, 2005). The structure comprises a cyclic peptide made of 7 amino acids to which 2 sugars are bound (Kang & Park, 2015). Attachment of the antibiotic to its target occurs through the formation of 5 hydrogen bonds using the peptidic backbone of the drug. Other drugs such as oritavancin have additional chlorine or sugar attached to the backbone, which enables it to bind more efficiently to the target (Allen & Nikas, 2003). The first glycopeptide to be used clinically was vancomycin. In its structure shown in figure 8 vancomycin has proteinogenic amino acids i.e (Tyrosine, Leucine, Asparagine, Alanine, and Glutamine) and nonproteinogenic amino acid residues i.e (4-hydroxyphenylglycine, 3, 5-dihydroxyphenylglycine, and β-hydroxytyrosine).

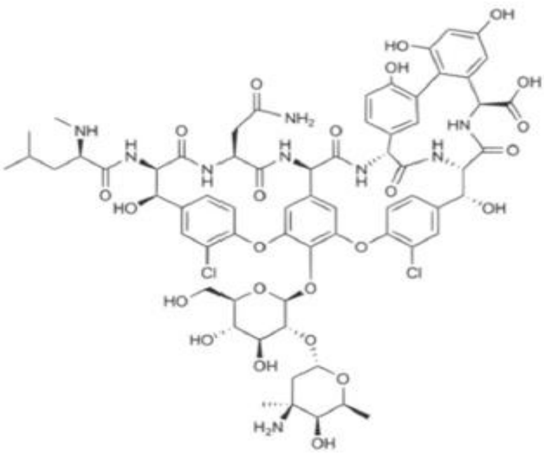

Figure 8: Structure of Vancomycin. The structure comprises a cyclic peptide (Kang & Park, 2015).

h) Aminoglycosides

Streptomycin commonly used against *Mycobacterium tuberculosis* was the first drug to be discovered in this family in 1943 and was obtained from soil actinomycetes (Mahajan & Balachandran, 2012). The structure is made of 3-amino sugars connected by glycosidic bonds as shown in figure 9. Their mode of action involves inhibition of protein synthesis in bacteria through binding to ribosomal subunits (Peterson, 2008). They have a broad spectrum of antibacterial activity and against aerobic gram-negative rods and certain gram-positive bacteria. Other infections treated by the drug include bubonic plague, tularemia, and tuberculosis (Talaro & Chess, 2008). Due to toxicity associated with streptomycin, a search for new members of the aminoglycosides led to discoveries of new drugs such as gentamicin, neomycin, tobramycin, and amikacin. Gentamicin is less toxic and is popularly used for treating infections caused by gram-negative rods such as *Escherichia, Pseudomonas, Shigella*, and *Salmonella* while tobramycin particularly is used in treating *Pseudomonas* infections in cystic fibrosis patients (Gilbert, 2000).

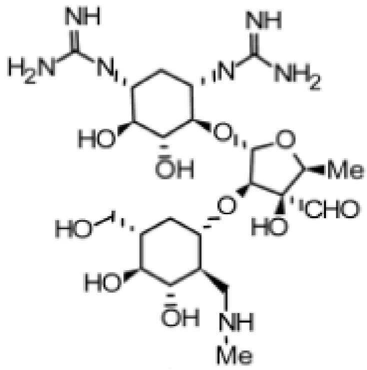

Figure 9: Structure of streptomycin. The structure is made of 3-amino sugars connected by glycosidic bonds (Loomans *et al.,* 2003).

2.4.1.1 Antibiotic resistance

The use of antibiotics has been accompanied by the rapid appearance of resistant strains, with more than 20,000 potential resistance genes of nearly 400 different types, predicted in the bacterial genome sequences (Liu & Pop, 2009). There is a strong correlation between antibiotic use in the treatment of diseases caused by gram-negative pathogens, such as *Escherichia coli*, *Salmonella enterica*, and *Klebsiella pneumonia*, and antibiotic resistance development to the β-lactam class of antibiotics over the past half-century (Davis & Davis, 2010). Antibiotic resistance to cephalosporin used for treating gonorrhea has been reported in Austria, Australia, Canada, France, Japan, Norway, Slovenia, South Africa, Sweden, and the United Kingdom (WHO, 2014).

The resistance of bacteria to various antibiotic treatments has been contributed by social and technical factors that enhance the spread of resistant genes, together with biochemical and genetic mechanisms of these organisms (Davis & Davis, 2010). Enhanced transmission of resistant genes has been contributed by extensive use of antibiotics in humans, veterinary medicine (Blackman, 2002), agriculture (Angulo *et al.,* 2004), and aquaculture (Reilly & Kaeferstein, 1997). The nutritive antibiotic treatment of farm animals amounts to half of the world's antibiotic output and has contributed majorly to the spread of antibiotic-resistant bacteria. This is because antibiotic-resistant bacteria from poultry, pigs, and cattle have found their way to the food supply and can be found in human food (Wegener, 2003), colonize the human digestive tract, and transfer resistance genes to human commensals.

Antibacterial resistance is associated with increased healthcare costs, prolonged hospitalization, and escalating morbidity and mortality in both developed and developing countries (Gulen *et al.,* 2015). In the US, an estimated $20 billion has been recorded to be lost due to antibacterial resistance with about $35 billion annually being lost in terms of productivity in health care systems because of antibiotic resistance (Ventola, 2015). It is approximated that by 2050, 10 million deaths will be as a result of antimicrobial resistance and about 100 trillion USD of the world's economic outputs lost to the drain if measures are not put in place to tame this threat (WHO, 2017).

2.4.1.2 Mechanism of bacterial antibiotic resistance and development

Because of differences in the structure of both gram-negative and gram-positive bacteria, there exist variations in mechanisms of resistance to various antibiotics by various bacteria. The presence of Lipopolysaccharides layer in gram-negative bacteria prevents the entrance of certain molecules thus allowing the bacteria to possess innate resistance to certain antimicrobial agents (Blair *et al.,* 2014). Certain bacteria such as Mycoplasma and related species which lack cell wall are intrinsically resistant to all drugs including β-lactams and glycopeptides that target the cell wall while gram-positive bacteria do not possess an outer membrane hence restriction of drugs is not common (Bébéar & Pereyre, 2005).

Gram-negative bacteria with large outer membranes possess porin channels that allow access to hydrophilic molecules. Mutations that cause changes within the porin channel leading to resistance to imipenem and certain cephalosporins have been observed in *E. aerogenes*. The resistances of *Neisseria gonorrhoeae* to β-lactams and tetracycline have also been associated with porin mutations (Thiolas *et al.,* 2004).

Other mechanisms used by the bacterial to develop resistance to various drugs include drug inactivation and drug efflux pumps. Drug inactivation entails actual degradation of the drug as observed in β-lactamases or transfer of chemical groups such as acetyl, phosphoryl, and adenyl to the drug leading to inactivation (Blair *et al.*, 2015). Acetylation is commonly used against aminoglycosides, chloramphenicol, streptogramins, and fluoroquinolones (Blair *et al.*, 2015). Drug efflux pumps are chromosomally encoded genes that function to rid the bacterial cell of toxic substances. They include MacB that extrudes macrolide drugs and EmrB, which extrudes nalidixic acid in *E. coli* (Jo *et al.*, 2017).

2.4.2 Plants as a source of antibacterial agents

Despite fungi, bacteria, and plants having the ability to synthesize secondary metabolites that possess antibacterial properties and other bio-efficacies, plants remain to be the major source of bioactive compounds because of greater chemical diversity than other organisms (Haruna & Yahaya, 2021). Berdy (2005) reports that more than a million compounds have been discovered from natural sources and among them, 50-60% is of plant origin and 5% were synthesized by micro-organisms. Approximately 28% of all new chemical entities launched onto the market between 1981 and 2002 were derived from plants (Newman *et al.*, 2003).

Plant extracts have been used in the treatment of diseases affecting humankind and livestock since time immemorial. Information about these plants is well documented with some of the plants currently in use by some communities still up-to-date (Rios & Recio, 2005). Roughly, 80% of the world's population utilizes herbal medicine, which has compounds obtained from medicinal plants. Consequently, such plants should be researched to better understand their properties, safety, and efficiency (Munuswamy *et al.*, 2013).

The bioefficacy of plant extracts has been investigated by several researchers. Ethanol extract of *D. adscendens* was reported to have antipsychotic-like activities in mice (Giovannini *et al.,* 2017). Antibacterial properties have been observed in the *Darlingtonia californica*, *Proboscidea louisianica*, *Alnus barbata*, and *Botrychium multifidum (*Hotti *et al.,* 2017*).* Ethanolic extract of *Cryptolepis sanguinolenta* and *Terminalia macroptera* showed antimicrobial activity against *C. jejuni* and *Campylobacter* spp (Hlashwayo *et al.,* 2020) whereas the antibacterial activity of *Tagetes erecta* flower was observed on *MSSA*, *MRSA*, *K. pneumonia*, and *P. aeruginosa* with MIC of 0.78 mg/ml, 3.13 mg/ml, 1.25 mg/ml, and 2.5 mg/ml respectively (Trinh *et al.,* 2020).

2.4.2.1 Secondary metabolites from plants and their efficacy

Various studies have shown secondary metabolites from plants do possess several biological activities, hence providing the scientific base for the use of herbal medicine in many ancient communities. Some of the efficacy includes antibacterial, antifungal, and antiviral. As a result, they can protect plants from pathogens. Further, these metabolites constitute important UV absorbing compounds, thus preventing serious leaf damage from the UV light. Based on their chemical structures, secondary plant metabolites are classified into different classes (Hussein & El-Anssary, 2017).

a) Saponins

Approximately 500 plants belonging to at least 90 different families have been reported to have saponins (Hussein & El-Anssary, 2017). They obtain their name from the soapwort plant Saponaria and have distinctive foaming characteristics (Sen *et al.,* 1998). They occur naturally as surface-active glycosides mostly produced by plants, lower marine animals, and some bacteria (Yoshiki *et al.,* 1998). All parts of plants i.e leaves, stems, roots bulbs, flowers, and fruits have been reported to contain these substances. Nevertheless, many species such as *Digitalis purpurea* (foxglove), *Dioscorea villosa* (wild yam), *Eleutherococcus senticosus* (Siberian ginseng), *Gentiana lutea* (gentian), *Glycyrrhiza* spp. (licorice) and *Panax ginseng* (Korean ginseng) tend to have saponins concentrated in the roots (Hussein & El-Anssary, 2017).

The basic structure of saponins entails a sugar moiety (glucose, galactose, glucuronic acid, and xylose) attached to a glycoside linkage and which is attached to a hydrophobic aglycone (sapogenin) which can be a triterpenoid or a steroid (Fenwick *et al.,* 1991). The variability of the aglycone structure and the position of attachment of the moieties in the aglycone create complexity in the saponins structure (Fenwick *et al.,* 1991). The combination of the nonpolar sapogenin and the water-soluble side chain enables saponin to foam (Francis *et al.,* 2002). Saponins possess various pharmacological properties which include antitumor, molluscicidal (Escalante *et al.,* 2002), spermicidal, sedative, expectorant for example Glycyrrhizin from *glycyrrhizae radix* is useful as an expectorant. Other properties include treatment of chronic hepatitis and cirrhosis, anti-inflammatory properties, analgesic properties, insecticidal (Morrissey & Osbourn, 1999), and antifungal (Delmas *et al.,* 2000) activities.

b) Alkaloids

They are among the largest single class of plant secondary metabolites with about 5500 known alkaloids. Their structure entails heterocyclic nitrogen compounds derived from amino acids such as tryptophan, tyrosine, and lysine. They have been classified into various groups based on biosynthetic intermediates and heterocyclic ring systems. These include indole, piperidine, tropane, purine, pyrrolizidine, imidazole, quinolizidine, isoquinoline, and pyrrolidine alkaloids (Kaur & Arora, 2015).

They have complex chemical structures with a long biosynthetic pathway. They derive their name from the word “alkaline” hence are organic bases that form salts with acids and when soluble they give alkaline solutions (Roy, 2017). Alkaloids are believed to play defensive roles in the plants against herbivores and pathogens hence approximately 20% of plants have been reported to have alkaloids (Roy, 2017). Many alkaloids have been in use for hundreds of years in medicine and currently, it is still among prominent drugs. Papaverine isolated from *Papaver somniferum* possesses inhibitory effects on several viruses while indoquinoline from *Cryptolepsis sanguinolenta* possesses activity against some gram-negative bacteria and yeast. Quinine, an alkaloid, is famous for its antimalarial properties against the malaria parasite (Staba & Chung, 1981). Other properties of alkaloids incude antiplasmodial (Frédérich *et al.,* 2002), anticorrosive (Capasso *et al.,* 2002), antioxidative (Czapski *et al.*, 2015), antibacterial (Karou *et al.*, 2005), anti-HIV (Zhang *et al.*, 2015) and insecticidal activities (Ge *et al.*, 2015).

c) Flavonoids

These are polyphenols majorly derived from plants and broadly distributed in foods and beverages. They form the major group of polyphenolic compounds, occur both in the free form (aglycones), and are bound to carbohydrates as glycosides. All flavonoids do have a basic C6-C3-C6 phenyl-benzopyran backbone (Harnafi & Amrani, 2007). The position of the phenyl ring in relation to the benzopyran moiety leads to the broad separation of these compounds into various classes. These classes include flavones, flavonols, flavanones, catechins, isoflavones, and anthocyanidins (Nijveldt *et al.*, 2001). They are popularly known for their ability to scavenge oxygen-derived free radicals owing to the presence of hydroxyl groups hence they act as antioxidants (Nijveldt *et al.,* 2001).

The exceptional antioxidant properties of these substances are attributed to the presence of hydroxyl groups in positions 3' and 4' of the B ring. This offers high stability to the formed radical by taking part in the displacement of the electron, and a double bond between carbons C_2 and C_3 of the ring C in conjunction with the carbonyl group at the C_4 position. This allows the displacement of an electron possible from ring B. Moreover, free hydroxyl groups in position 3 of ring C and position 5 of ring A, besides the carbonyl group in position 4 are also vital for the antioxidant activity of these compounds (Sánchez-Moreno, 2002).

Nonetheless, the effectiveness of the flavonoids decreases with the substitution of hydroxyl groups for sugars hence glycosides are fewer antioxidants than their corresponding aglycones (Rice-Evans *et al.,* 1996). They have also been reported to enhance the vaso-relaxant process (Bernatova *et al.*, 2002). Other biological activities include antiviral, antibacterial, antifungal, antiproliferative, antiallergic, antidiabetic, antiviral, antibacterial, antifungal, antiproliferative, anticarcinogenic and hepatoprotective, (Cowan, 1999; Cushnie & Lamb, 2005).

d) Terpenoids

Terpenoids are also known as terpenes. They are a diverse group of naturally occurring compounds widely distributed in plants. These plants include tea, thyme, cannabis, Spanish sage, and citrus fruits e.g., lemon, orange, mandarin (Cox-Georgian *et al.*, 2019). They form the major constituent of essential oils in plants and are classified as mono, di, tri, tetra, and sesquiterpenes depending on the number of isoprene units (Mahizan *et al.,* 2019). Besides forming essential oils in plants, these compounds have been noted to play several roles in plants, which include thermoprotectant, signaling functions, pigment formation, and flavoring agents (Yang *et al.,* 2012).

Terpene synthases are the key enzymes involved in the formation of terpenes. A large number of different terpene synthases have resulted in a diversity of terpenoids. Besides, a large number of different terpene synthases produce multiple products (Degenhardt *et al.*, 2009). The biosynthetic pathway for terpenoids occurs within the cytosol of plants and in other living systems as well (Degenhardt *et al.*, 2009). Some of the pharmacological properties of terpenoids are anticancer, antimicrobial, antifungal, antiviral, antihyperglycemic, analgesic, anti-inflammatory, and antiparasitic (Franklin *et al.,* 2000). Moreover, they are also used to enhance skin penetration, prevent inflammatory diseases, and hence have large-scale application in a variety of treatment drugs (Franklin *et al.,* 2000).

e) Essential oil

Essential oils (EOs) are generally volatile compounds produced by aromatic plants, which represent 10% of the plant kingdom and are associated with a strong odour (Adorjan & Buchbauer, 2010). The volatile components present in the oil are classified as terpenes (monoterpenes-C10, sesquiterpenes-C15, and diterpenes-C20), phenolic-derived aromatic, and aliphatic components (Abad *et al.*, 2012). Various plant parts such as flowers, leaves, stems, fruits, seeds, and roots harbor essential oils, which are stored in special brittle secretory structures, such as glands, secretory hairs, and trichomes (Combrinck *et al.,* 2007). In plants, the total essential oil composition is generally minimal, and hardly does it surpass 1% (Bowles, 2003).

Many factors affect the chemical composition of essential oils resulting in same species of plants producing similar essential oils but different chemical composition. These factors are the genetic composition of the plant, climatic conditions, and the period when plant material was collected for oil extraction (Andrade *et al.,* 2011). This is because the factors influence the biochemical synthesis of essential oils in a given plant. The common method for extraction of the oil involves hydro-distillation using a Clevenger-type apparatus (Abad *et al.,* 2012).

These oils have a broad spectrum of bioactivity due to the presence of several active ingredients. These ingredients are the low molecular weight volatile components that enable them to diffuse quickly through the skin membranes and thus reaching target sites quickly (Gutierrez *et al.,* 2009). These low molecular weight substances also make the essential oils to be extremely concentrated with one drop of the oil being reported to have 40 million trillion molecules thus the reason for their amazing bioactivity (Stewart, 2005). The components also work through various modes of action resulting in various bioefficacies, which include antibacterial, antifungal, antiviral, antiparasitic, and insecticidal (Abad *et al.,* 2012).

2.4.2.2 Pyrethrum plant, *Chrysanthemum cinerariaefolium*

It is a perennial plant with white-yellow flowers as shown in figure 10. The plant grows in highlands with an altitude of 2400-3000 m above sea levels, rainfall between 1000-1200 mm, and well-drained fertile soils (Bisht *et al.,* 2009). The plant produces pyrethrins as major phytochemical, widely used for the production of natural insecticide (Hitmi *et al.,* 2000).

Pyrethrins are mainly concentrated in the flower heads with 93.7% in achenes and minor quantities in disc florets (2.0%), ray florets (2.6%), and receptacles (2.6%) (Essig & Zao, 2001b). Pyrethrins are effective as insecticides since they have low mammalian toxicity, environmentally safe due to rapid degradation on exposure to light and air, have antifeedant properties on insects, repellency effects, and rapid knock-down on a wide range of flying insects (Grdiša *et al.,* 2009). They are formulated with stabilizers and synergists to enhance their stability in air and light (Hitmi *et al.,* 2000). Previous studies on *C. cinerariaefolium* has also shown that pyrethrin esters isolated from the plant inhibited multiple-drug resistant (MDR) *Mycobacterium tuberculosis* at concentrations of 33 µg/ml and 100 µg/ml (Rugutt *et al.,* 1999). Scanty studies are available on antibacterial potential of *C. cinerariaefolium* hence there is need to investigate.

Figure 10: Image of *C. cinerariaefolium* (Pyrethrum) flowers (KALRO, 2019).

There are other phytochemicals besides the pyrethrins that have been identified in the pyrethrum plant. The pyrethrum essential oil is reported to have (E)-β-farnesene, germacrene D, isocaryophyllene, spathylenol, trans-chrysanthemumic, β-cubebene, δ-cadinene, α-copaene, and δ-nerodilol (Saggar *et al.,* 1997). Others are sesquiterpene lactones (11R)-11,13-dihydrotatridin-A, (11R)-11,13-dihydrotatridin-B, (11R)-6-O-β-D-glucosyl-11,13-dihydrotatridin-B, tatridin-A, tatridin-A, and tatridin-B (Galal, 2001).

2.4.3 Silver as an antibacterial agent

The use of silver in the treatment of various infections dates back to ancient civilizations. In recent years, this material has found its way back as a therapeutic option due to the increasing prevalence of bacterial resistance to conventional antimicrobials agents (Sim *et al.,* 2018). Silver is a shiny transition element that is soft and has the highest reflectivity of all metals (Lansdown, 2010). The bioactive form of silver is the monoatomic ionic state (Ag^+) formed when silver is solubilized in aqueous environments. The same form appears in ionic silver compounds such as silver nitrate and silver sulfadiazine, which are frequently used to treat wounds. Besides the silver ions, the native form of the silver nanocrystalline form (Ag^0) is also biologically active (Fong & Wood, 2006).

Due to its renowned antibacterial properties, silver has found applications in various fields such as the medical field, textiles, cosmetics, and even domestic appliances among other areas (Lansdown, 2010). In the medical field, devices that are normally implanted in the human body are usually coated with silver nanoparticles for antimicrobial effects. These devices include vascular catheters, bone implants, biliary duct brackets, and invasive surgical tools such as medical-grade needles (Sim *et al.,* 2018). Another application in the medical field is ready packed medical apparatus that sterilizes itself using silver upon the opening of the package. This is by creating a vapor that triggers a silver-containing hydrophilic surface coating. Moreover, silver has been the key component in dental amalgam fillings for more than one hundred years (Sim *et al.,* 2018).

In topical treatments, several topical gels with different formulations of silver have been produced. Silver in the form of 0.5% silver nitrate solution and silver sulfadiazine cream was first used to treat burn wounds in the 1960s (Nherera *et al.,* 2017). In personal care products and cosmetics such as cream, aqueous lotions, or hydrogel medium, manufacturers incorporate silver colloids into these products since they do not precipitate and separate the products thus an added advantage of acting as a preservative (Sim *et al.*, 2018). For agriculture use, silver has been incorporated in nylon ropes that are used to tie down plants hence preventing from decaying after time because of bacterial biofilm formation (Ingle *et al.,* 2010).

The use of silver products for agricultural purposes must be with caution to avoid any impact on microbial flora and symbiosis. This is because the growth of healthy crop plants depends so much on the formation of symbiotic relationships with microbes around the roots such as nitrifying bacteria and *mycorrhiza* (Hayat *et al.*, 2010). In fact, contact of bioactive silver with nitrifying bacteria has been reported to hinder the formation of symbiotic channels (Choi & Hu, 2008).

The antimicrobial application of silver in domestic products started in ancient times during the Phoenician, Macedonian, and Persian empires (Alexander, 2009). Families of these empires and other higher socioeconomic status used silver vessels and plates frequently until they developed bluish skin discolorations a condition known as *argyria*, an affliction that led to them being termed 'blue blood' (Alexander, 2009). Today many domestic appliances are coated with silver to create bacteria-free surfaces. These appliances include automated bathtubs, laundry washing machines, air purifiers with silver filters, and refrigerators.

Silver is believed to cause bactericidal activity by forming a complex with DNA. This follows an in vitro study in which radio-labeled silver sulfadiazine (SSD) was used (Fox and Modak, 1974). Further studies illustrated that silver causes the precipitation of DNA within bacteria (Feng *et al.,* 2000). The metal is also believed to exert bactericidal activity by binding strongly with membranes and cell wall proteins probably because of its interaction with thiol groups on enzymes (Jung *et al.*, 2008).

2.5 Nanotechnology

It is an important field of modern research that is characterized by the design, synthesis, and manipulation of particles that have a size ranging from approximately 1-100 nm and in one dimension. These particles are the nanomaterials that include the nanoparticles and the nanostructures (Kumara *et al.,* 2014). Due to their small sizes, the nanomaterials possess novel physiochemical and biological properties, such as enhanced reactive area and the ability to cross-cell and tissue barriers (Nikam *et al.,* 2014). Besides, the nanoparticles and nanostructures have gained popularity in technological advancements because of their tunable physicochemical characteristics such as melting point, wettability, electrical and thermal conductivity, catalytic activity, light absorption, and scattering causing them to have enhanced performance over their bulk counterparts (Jeevanandam *et al.*, 2018).

The nanomaterial acts as a narrow bridge between molecules /atoms and bulky materials. They include clusters, quantum dots, nanocrystals, nanowires, and nanotubes. A collection of nanostructures creates an array, assemblies, and superlattices of the individual nanostructures (Sharma *et al.,* 2009). The uniqueness of the nanomaterial in terms of structural characteristics, energetics, response, dynamics, chemistry, and ability to be manipulated, has enabled these materials find widespread application in various fields. They have therefore offered a solution to challenges affecting health care, cosmetics, food and feed, environmental health, mechanics, optics, biomedical sciences, chemical industries, electronics, space industries, drug-gene delivery, energy science, optoelectronics, catalysis, single-electron transistors, light emitters, nonlinear optical devices, and photoelectrochemical applications (Donega, 2011, Guglielmo, 2010).

2.5.1 Nanoparticles

The two major classes in which nanoparticles are broadly grouped are organic and inorganic nanoparticles. The organic nanoparticles are the carbon nanoparticles (fullerenes) whereas; the inorganic nanoparticles are magnetic nanoparticles, noble metal nanoparticles (like gold and silver), and semi-conductor nanoparticles (like titanium oxide and zinc oxide). The nanomaterial in the fullerenes is made of a globular hollow cage similar to allotropic forms of carbon. Due to their electrical conductivity, high strength, structure, electron affinity, and versatility they have attracted remarkable commercial interest (Astefanei *et al.,* 2015).

A lot of attention in the medical field has been focused on inorganic nanoparticles made of noble metals i.e (gold and silver nanoparticles), because of their superior properties. They are extensively available, have good biocompatibility, good carriers of targeted drug delivery, controlled drug release of drugs, and rich functionality. Due to their nano size, they are easily used as chemical imaging drug agents and drugs (Xu *et al.*, 2006).

Particle size and size distribution are important factors in nanoparticle production. These properties have an effect on in vivo distribution, biological fate, targeting ability of nanoparticle system, and toxicity. Moreover, drug release, drug loading, and stability of nanoparticles are also affected by particle size (Panyam & Labhasetwar, 2003).

Due to their small size, nanoparticles have higher intracellular uptake than microparticles. As a result, they are available to an extensive range of biological targets. Nanoparticles that had a size of 100 nm had 2.5 fold increased uptake than 1 μm microparticles and 6 fold more uptake than 10 μm microparticles in a Caco-2 cell line (Desai *et al.,* 1997). Besides, nanoparticles also can cross the blood-brain barrier after the opening of tight junctions by hyperosmotic mannitol. This provides sustained release of drugs for the treatment of hard-to-treat diseases like brain tumors. An example of such nanoparticles is the Tween 80 coated nanoparticles that have been shown to cross the blood-brain barrier (Kreuter *et al.,* 2003).

There is a relationship between particle size and drug release. This is because smaller nanoparticles have larger a surface area as a result most of the drug that would associate with it would be near the particle surface, leading to fast drug release while larger particles have large cores hence drugs are normally encapsulated leading to slowly diffuse out (Redhead *et al.,* 2001). Despite the advantage associated with smaller nanoparticles, smaller nanoparticles have an increased risk of aggregation during storage and transportation, thus it is always a challenge to formulate nanoparticles with the smallest size possible and maximum stability (Mohanraj & Chen, 2006).

2.6 Silver nanoparticles (Ag NPs)

In nanotechnology, silver nanoparticles are one of the promising products currently utilized in about 25% of all consumer products. This is due to their unique characteristics such as small size i.e less than 100 nm and peculiar chemical and physical properties (Vance *et al.*, 2015).

2.6.1 Properties of silver nanoparticles

Generally, particle size affects the functionality of silver nanoparticles. Smaller nanoparticles have a larger surface area to volume ratio hence exhibit more bioactivity than larger-sized particles (Lu *et al.,* 2013). In a macrophage cell line, 15 nm Ag NPs were reported to generate more ROS than 55 nm Ag NPs (Carlson *et al.,* 2008). Particle size is affected by the concentration of both the precursor metals salts used in the synthesis and the reducing agent. Larger nanoparticles were generated by increasing the concentration of Ag NO_3 from 2.5 to 15 M. Similar observation was noted when the concentration of the polysaccharide (reducing agent) was increased (Pandey *et al.,* 2012).

Diverse shapes of Ag NPs can be produced depending on the method of synthesis. Green synthesis mostly generates diverse shapes such as spherical, oval, rod, and flower-shaped Ag-NPs whereas chemical synthesis mostly leads to the production of spherical shapes (Akter *et al.,* 2018). The shape of the Ag-NPs determines the cellular uptake mechanism hence influence cytotoxicity. Adverse effects were not observed when A549 cells were subjected to spherical Ag NPs while negative outcomes were induced by wire-shaped nanoparticles (Stoehr *et al.,* 2011). Besides, truncated triangular nanoparticles showed the strongest biocidal action against the gram-negative bacterium *Escherichia coli* in comparison with spherical and rod-shaped nanoparticles (Pal *et al.*, 2007).

Optical properties of the Ag NPs determined using the UV-Vis spectroscopy include the Localized Surface Plasmon Resonance (SPR). The optical properties are of immense interest due to the strong coupling of Ag NPs to specific wavelengths of incident light at a certain SPR. The plasmonic properties for larger particles have localized SPR wavelength in the red region of the visible spectrum while small nanospheres have short SPR wavelengths in the violet and blue regions of the visible spectrum (Lu *et al.*, 2013). The plasmonic properties can be utilized for bio-sensing. An example is the utilization of plasmonic coupling between single pairs of silver and gold nanoparticles in tracking DNA hybridization (Sönnichsen *et al.*, 2005).

Due to advances in technology, several techniques are available for ascertaining these Ag NPs characteristics. Particle size is ascertained using the Dynamic Light Scattering technique (DLS). In DLS, light from a laser interacts with the moving particles in the solution at a given frequency that results in the scattering of the light. The scattered light results in a change in frequency of a given light, which is thus proportional to particle size in the solution (Carvalho *et al.,* 2017). Scanning Electron Microscope (SEM) and Transmission Electron microscope (TEM) also give high-resolution images of the surface of the desired sample hence can be used to ascertain, particle size, morphology, and shape of the nanoparticles (Hamouda *et al.*, 2019).

Fourier transmission infrared spectroscopy uses infrared light to determine the surface composition of Ag NPs formed through stabilization and reduction by various metabolites such as the phytochemicals. This is because it measures vibration characteristics of chemical functional groups on the sample, as these functional groups tend to absorb infrared radiation in a specific wavenumber range (Elamawi *et al.,* 2018). Optical properties such as the SPR is determined using UV-Vis while X-Ray Diffraction(XRD) gives phase composition of a sample, crystal structure, texture, or orientation. This is because X-rays will give diffraction patterns that provide information about the atomic arrangement within the Ag NP crystals (Mehta *et al.,* 2017).

2.6.2 Synthesis of silver nanoparticles

There are various methods used for synthesizing silver nanoparticles. These methods are the physical, chemical, and biological.

2.6.2.1 Physical method

This method involves the evaporation-condensation approach and the laser ablation technique (Iravani *et al.,* 2014). These two methods can produce Ag NPs in high numbers in absence of toxic chemicals that threaten human health and the environment. The greatest disadvantage associated with these methods is agglomeration due to the absence of stabilizers/capping agents. Moreover, both methods consume a lot of power, need complex equipment, require a longer duration, and increased operating costs (Lee & Jun, 2019).

In the evaporation-condensation technique, the reaction is carried out using a tube furnace with the target material being kept within a boat centered at the furnace. The sample is then vaporized into a carrier gas (Kruis *et al.,* 2000). This technique has been used to synthesize various nanospheres from various materials such as gold, silver, and lead sulphide (Kruis *et al.,* 2000). Nonetheless, the evaporation-condensation technique has several limitations, which include; the tube furnace occupies a large space, utilizes high energy increasing the surrounding temperature, and for thermal stability a lot of time is required. To overcome the problems a ceramic heater can be used (Jung *et al.,* 2006).

Laser ablation of metallic bulk materials in solution could also be utilized in the synthesis of Ag NPs (Sylvestre *et al.,* 2004). Many factors such as the wavelength of the laser impinging the metallic target, the duration of the laser pulses, the ablation time duration, and the effective liquid medium in the presence or absence of surfactants affect the ablation efficiency and features of the produced nanosilver (Tarasenko *et al.,* 2006).

2.6.2.2 Chemical method

It is the frequently used method. It involves the synthesis of metallic NPs as a colloidal dispersion in an aqueous solution or organic solvent via reduction of their silver salts. The method utilizes different reducing agents such as sodium citrate, ascorbate, sodium borohydride (NaBH4), elemental hydrogen, polyol process, tollens reagent, N, N-dimethylformamide (DMF), and poly (ethylene glycol)-block copolymers to reduce silver ions (Ag^+) in aqueous or non-aqueous solutions (Goulet & Lennox, 2010). This lead to the formation of metallic silver (Ag^0), which eventually undergo agglomeration into oligomeric clusters resulting in the formation of metallic colloidal silver nanoparticles (Wiley *et al.,* 2005).

Stabilizing dispersive nanoparticles is essential during nanoparticle synthesis. As a result, stabilizers that can be absorbed onto the surface of Ag NPs are usually used hence preventing agglomeration (Bai *et al.*, 2007). Common stabilizers are the capping agents/surfactants such as chitosan, oleylamine gluconic acid, cellulose, or polymers, such as polyN-vinyl-2-pyrrolidone (PVP), polyethylene glycol (PEG), polymethacrylic acid (PMAA), and polymethylmethacrylate (PMMA) are used (Pillai & Kamat, 2004).

Electrostatic or steric repulsion is utilized to achieve stabilization via capping agents. An example is the achievement of electrostatic stabilization via anionic species, such as citrate, halides, carboxylates, or polyoxoanions that adsorb or interact with Ag NPs to impart a negative charge on Ag NPs surface (El Badawy, 2012). In comparison to using citrate ions, which gives a negative charge, branched polyethyleneimine (PEI) creates an amine-functionalized surface with a highly positive charge (Salih *et al.*, 2019). Other capping agents also provide extra functionalities. For example, Polyethyleneglycol (PEG)-coated nanoparticles demonstrate good stability in extremely concentrated salt solutions, whereas lipoic acid-coated particles with carboxyl groups can be utilized for bioconjugation (Lee & Jun, 2019). One limitation with the chemical method is the presence of toxic chemicals absorbed on the surface that may have adverse effects in the medical applications hence alternative methods of synthesizing the nanoparticles such as green synthesis are being advocated (Rafique *et al.,* 2017).

2.6.2.3 Green synthesis of silver nanoparticles

The current methods i.e physical and chemical methods utilized in the production of Ag NPs are expensive, toxic, and non-environment friendly. To overcome these challenges, researchers have devised alternative methods for synthesis that employ naturally occurring reducing agents (green chemistry). These agents are present in biological entities, such as fungi, bacteria, and plant extracts in the synthesis of silver nanoparticles (Ahmed *et al.*, 2015). The use of green synthesis in the production of Ag NPs could be a promising method in replacing complex physiochemical syntheses. This is because the method is free from toxic chemicals, economical, efficient, and hazardous products are absent as it involves natural capping agents for the stabilization of Ag NPs (Abou El-Nour *et al.,* 2010).

a) Synthesis of silver nanoparticles using bacteria

Bacteria have been known to have the potential to synthesize Ag NPs intracellular using components present inside the cells, which serve as both reducing and stabilizing agents (Patra *et al.*, 2015). The ability of bacteria to reduce nitrate to nitrite and ammonium is key in the synthesis of Ag NPs. This is possible courtesy of the reducing power of nicotinamide adenine dinucleotide phosphate (NADPH) that is utilized by nitrate reductase (Kalimuthu *et al.*, 2008). Hydroxyquinoline might act as an electron shuttle, transferring electrons produced during the reduction of nitrate to Ag^{2+} allowing conversion of Ag^{2+}ions to Ag^0 (Lee & Jun, 2019). Figure 11 shows the schematic diagram for synthesizing Ag NPs using bacteria.

Some examples of bacteria that have been reported to synthesize nanoparticles include *Bacillus licheniformis*, which used electrons released from NADH to drive the reduction of Ag^+ to Ag^0 leading to the formation of Ag NPs with the size range of 50 nm (Kalimuthu *et al.*, 2008). Another example is *Pseudomonas stuzeri* isolated from silver mine was used in the synthesis of Ag NPs in aqueous Ag NO_3 with a size of 200 nm (Klaus *et al.,* 1999). Moreover, synthesis of Ag NPs can also be done using naturally occurring reducing agents such as supernatants. For example, culture supernatants of *S. aureus* were used to synthesize Ag NPs (Nanda & Saravanan, 2009).

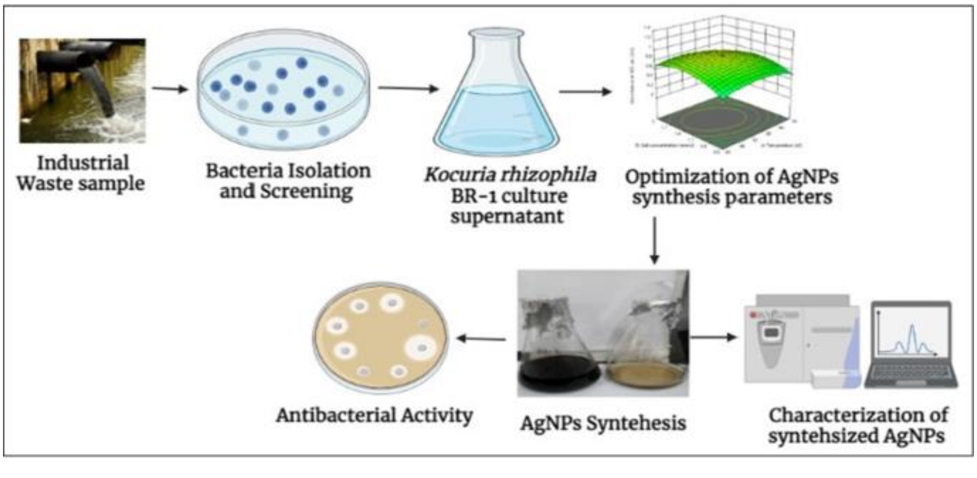

Figure 11: Shematic diagram showing synthesis of Ag NPs using bacteria (Kumar *et al.*, 2022).

b) Synthesis of silver nanoparticles using fungi

Fungi are considered the best nano-factories since they have the potential to synthesize large amounts of metallic NPs. This is due to their ability to secrete large amounts of proteins, high metal bioaccumulation capacity, high binding capacity, and intracellular uptake (Sastry *et al.,* 2003). The proposed mechanism responsible for the biosynthesis of Ag NPs by the fungi is that Ag^+ ions are first trapped on the surface of the fungal cells due to the electrostatic interaction between Ag^+ ions and negatively-charged carboxylate groups of the enzyme. The Ag^+ is then reduced by the enzymes present in the cell wall, leading to the formation of Ag nanoparticles (Vahabi *et al.,* 2011). It has been reported that extracellular enzymes such as naphthoquinones and anthraquinones facilitate the reduction (Ahmad *et al.,* 2003). A schematic diagram showing the biosynthesis of Ag NPs using fungi is shown in figure 12.

Several studies have reported on the ability of fungi to synthesize Ag NPs. Monodispersed Ag-NPs with an average size of 8.92 ± 1.61 nm were synthesized using fungus *Aspergillus flavus* (Vigneshwaran *et al.,* 2007). The fungus *Cladosporium cladosporioides* carried out the extracellular synthesis of Ag NPs with an average size of 10-100 nm (Balaji *et al.,* 2009). The exposure of an aqueous solution of Ag NO_3 to the fungal biomass belonging to fungus *Verticillium* resulted in the intracellular reduction of the metal ions to NPs that are spherical in shape and size range up to 25 ± 12 nm (Mukherjee *et al.,* 2001).

In comparison to other microorganisms, there is increased utilization of fungi since they are eco-friendly and easy to handle. Besides fungi such as white rot are non-pathogenic (Vigneshwaran *et al.,* 2006).

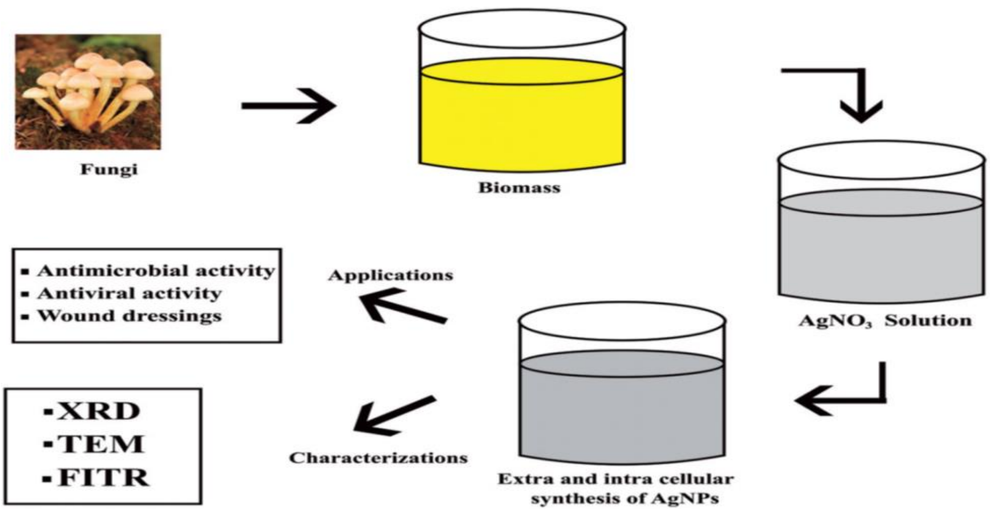

Figure 12: Steps involved in synthesis of Ag NPs using fungi (Rafique *et al*., 2017).

c) **Synthesis of silver nanoparticles using plants**

The use of plants in the synthesis of silver nanoparticles is a new trend that has come to the fore. The major benefits associated with the use of using plant extracts for silver nanoparticle synthesis are, they are easily available, safe, nontoxic in most cases, have broad varieties of metabolites that can aid in the reduction of silver ions, and are quicker than microbes in the synthesis since they do not need culturing (Huang *et al.,* 2007). Phytochemicals present in the plants are the main substances responsible for the reduction of metal ions to nanoparticles. These phytochemicals are terpenoids, flavones, ketones, alkaloids, aldehydes, amides, steroids, and carboxylic acids. In nanotechnology, these chemical entities act as reducing and capping/stabilizing agents in the synthesis of metal nanoparticles (Swarnalatha *et al.,* 2013). Figure 13 shows the schematic diagram for the synthesis of silver nanoparticles using plant extracts.

Numerous studies are displaying the use of plant extracts in the synthesis of nanoparticles. Among them, include the synthesis of silver nanoparticles by utilizing *Ananas comosus* (pineapple juice) as stabilizing and reducing agent, which led to the formation of spherical NPs with an average diameter of 12 nm (Ahmad & Sharma, 2012). The reduction of aqueous Ag NO_3 solution using extracts of *Neem* and *Triphala* led to the formation of Ag NPs that were spherical with particle size range 43 nm and 59 nm (Gavhane *et al.,* 2012). Peanut shell was used to synthesize silver nanoparticles that were spherical and oval with a size range of 10-50 nm (Velmurugan *et al.,* 2015). Silver nanoparticles with an average diameter of 20 nm were synthesized using the fruit extract of *Malus domestica* as a capping and reducing agent (Roy *et al.*, 2014).

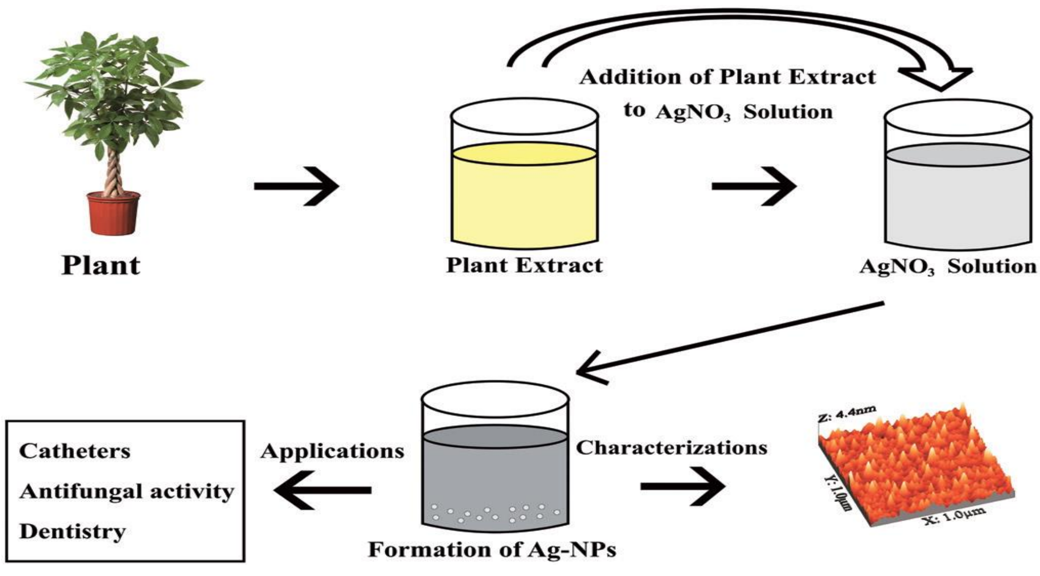

Figure 13: Shematic diagram showing synthesis of Ag NPs using plant extracts (Rafique *et al*., 2017).

2.6.3 Applications of silver nanoparticles (Ag NPs)

Silver nanoparticles have been widely used in the medical field therapeutically as antibacterial, antifungal, anti-viral, and anticancer agents. The antibacterial properties of Ag NPs have been reported in many studies. A concentration of (3.3-33 nM) inhibits *E. coli* and *S. aureus* (Shrivastava *et al.,* 2007). Ag NPs produced using *Cryptosporiopsis ericae* demonstrated activity against *S. aureus*, *E. coli*, and *E. faecalis* (Devi & Joshi, 2014). Antibacterial activity of Ag NPs synthesized using a*ctinomycetes* was observed against gram-negative and positive bacteria i.e *P. putida, K. pneumoniae, B. subtilis,* and *S. typhi* (Manivasagan *et al.,* 2013).

The mode of action of Ag NPs on bacteria has not been fully ascertained. Some of the proposed mechanism of action includes adhesion onto the membrane surface of microbial cells resulting in modification of the lipid bilayer. This leads to increased membrane permeability and intracellular penetration of Ag NPs. The penetrated Ag NPs would then damage the intracellular micro-organelles (i.e., mitochondria, ribosomes, and vacuoles) and biomolecules such as DNA, proteins, and lipids via the generation of reactive oxygen species (ROS) and free radicals (Lee & Jun, 2019).

The immense antibacterial properties of Ag NPs have made these molecules of great demand in comparison to other nanoparticles (Vance *et al.,* 2015). Some of the areas where antibacterial properties of Ag NPs have been exploited include production of wound dressing agents, food packaging materials (Li *et al.,* 2013), incorporation into water purification system (Thakare & Ramteke, 2017), coating of medical devices, household products, antiseptics in health care delivery, personal healthcare products (Tran & Le, 2013), and textile coatings (Von Goetz *et al.,* 2013). The anticancer properties of silver nanoparticles are associated with the anti-angiogenic and anti-proliferative properties of these nanoparticles (Rani *et al.,* 2009). The anti-proliferative property is a result of the ability of these molecules to cause DNA damage and chromosomal break. They also cause disturbance of ($Ca^{2+)}$ homeostasis leading to induction of apoptosis and cytoskeleton injury, which in turn block cell cycle and promote anti-proliferation of cancer cells (Zhang *et al.,* 2018).

Previous studies on the anti-viral activity of Ag NPs were reported by Elechiguerra *et al.,* (2005). He showed that Ag NPs ranging in size from 1 to 10 nm inhibited HIV-1 while 10-80 nm particles had the potential to inhibit other viral strains through binding to the outer proteins of the viral particles. Anti-fungal properties of Ag NPs against *Trichophyton rubrum*, *Trichophyton mentagrophytes*, and *Candida albicans* at different sizes and concentrations have been reported (Akter *et al.*, 2018).

Besides the therapeutic properties of Ag NPs, these particles can absorb and scatter light with great efficiency thus allowing individual Ag NPs to be imaged under dark-field microscopy or hyperspectral imaging systems (Liu *et al.*, 2012). As a result, these nanoparticles are promising fluorescent labels for imaging experiments (Zhang *et al.*, 2010). Examples of imaging done using Ag NPs include; bioimaging of cancer cells (Liu *et al.*, 2012) and detection of p53 in carcinoma cells (Zhou *et al.*, 2011).

2.6.4 Toxicity of silver nanoparticles

The mounting utilization of Ag NPs in day-to-day life has led to the release of these particles into the environment (Hedberg *et al.*, 2014). Ag NPs have been detected extensively in water and soil where they have been noted to accumulate in large quantities (Gottschalk & Nowack, 2011). Following an analysis of wastewater from a sewage treatment plant, Ag NPs with a size of 9.3 nm and a concentration of 1900 ng/L were observed (Hoque *et al.*, 2012).

Once in the environment, humans interact with these nanoparticles through the skin, lungs, and digestive tracts since these organs are in contact with the environment (Date *et al.*, 2016; Schneider *et al.*, 2009). The greatest susceptible routes are the lungs and digestive tract since the skin is a tougher organ acting as an external barrier against foreign substances in general. The use of injections and implants that have Ag NPs coating are other possible routes in which humans get exposed (Parnia *et al.*, 2017). As a result of their ultra-small sizes, Ag NPs can reach tissues and organs via circulatory and lymphatic systems resulting in a variety of health hazards.

Some of the drastic effects of silver nanoparticles include induction of toxic effects on the proliferation and cytokine expression by peripheral blood mononuclear cells (Shin *et al.*, 2007), ability to cross blood-testis barrier leading to inhibition of sperm production (McAuliffe & Perry, 2007) whereas oral toxicity studies on rats showed bile duct hyperplasia (Kim *et al.*, 2011). The disruptions of epithelial cell microvilli and intestinal glands were observed when Ag NPs (5–20 nm) were orally administrated for 21 days in mice (20 mg/kg of body weight) (van der Zande *et al.*, 2012). Moreover, accumulation of PVP-Ag NPs (14 nm) in various organs which include the intestines, the liver, the kidneys, the lungs, and the brain has been observed following oral administration in rats (Loeschner *et al.*, 2011).

The toxicity of silver nanoparticles has been reported to have a strong relationship with their physicochemical characteristics. The toxicity has been reported to be size-dependent (Karlsson *et al.,* 2009), shape-dependent (Oh *et al.,* 2010), aggregation or agglomeration-dependent (Abdelmonem *et al.,* 2015), and dose-dependent (Tiwari *et al.,* 2011).

Pertaining size, several studies have shown that the toxicity of Ag NPs is greater when smaller compared to large nanoparticles (Akter *et al.,* 2018). A study on the effect of different sizes of Ag NPs subjected to four cell lines (A549, HepG2, MCF-7, SGC-7901) by Liu *et al.,* (2010), observed that 5 nm Ag-NPs were more toxic than 20 and 50 nm Ag-NPs. A 20 nm citrate-coated Ag-NPs exhibited greater cytotoxicity than 110 nm Ag-NPs, furthermore, it generated acute neutrophilic inflammation in the lungs of mice in comparison with larger Ag NPs (Wang *et al.,* 2014).

The shape of the Ag NPs affects their cellular uptake mechanism, which in turn determines their cytotoxicity (Akter *et al.*, 2018). Silver Nanoparticles that were shaped as rods showed the highest uptake potential which was then followed by nanospheres, cylinders, and cubes are followed it, respectively (Gratton *et al.*, 2008). When Ag nanowires (length: 1.5-25 μm) and nanospheres (30 nm) were compared, it was found that the nano-wires were more toxic on alveolar epithelial cells (Stoehr *et al.,* 2011).

The concentration of NPs is another critical factor that affects toxicity. There is therefore a need to determine the minimum concentration level of NPs that can induce toxicity in various subjects (Akter *et al.*, 2018). In the majority of studies, Ag NPs showed cytotoxicity in a concentration-dependent manner. A concentration of 0.2 ppm Ag NPs reduced cell viability by 20% in RAW 264.7 cells, while 0.2 ppm 1.6 ppm of Ag NPs reduced viability by 40% (Park *et al.,* 2010). A similar trend was also witnessed in human Chang liver cells, whereby cell viability decreased in a concentration-dependent fashion (Piao *et al.,* 2011).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample collection

Pyrethrum flowers were obtained from Elgeyo-Marakwet County shown in figure 14 which is located at (latitude 00 10' to 00 52" N and Longitude 350 25" to 350 45" E) and has an altitude of 8389 m above sea level (http://www.kenyampya.com). It borders the counties of West Pokot to the north, Baringo County to the east, southeast and south, Uasin Gishu to the southwest and west, and Trans Nzoia to the northwest (http://www.kenyampya.com).

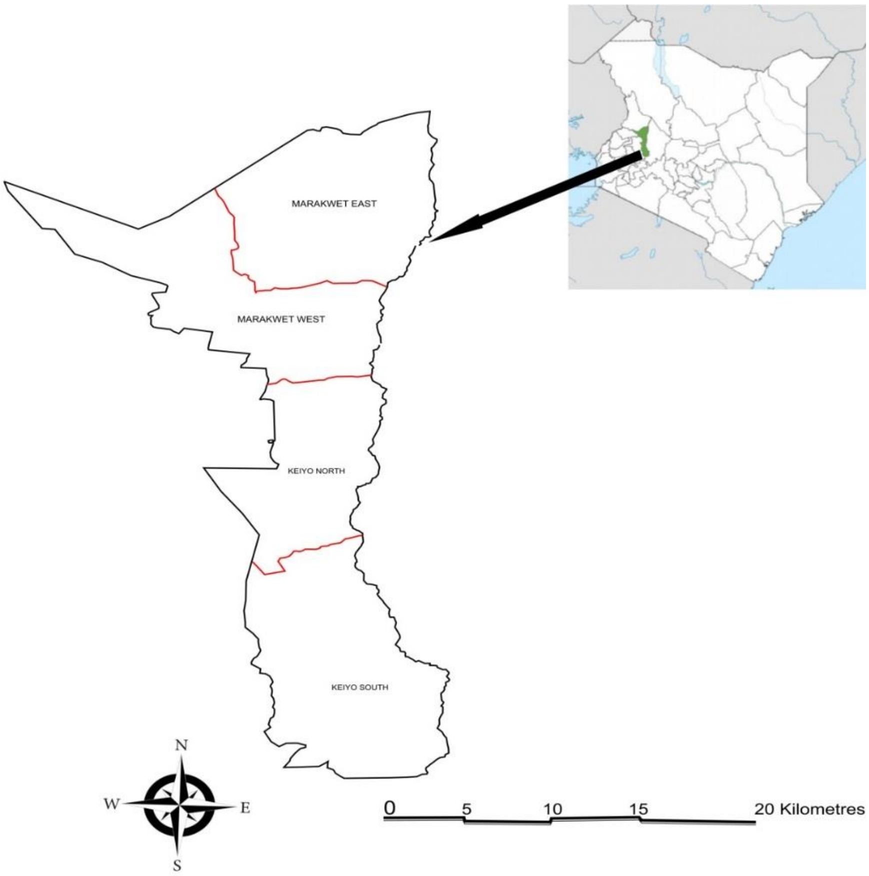

Figure 14: A map of Kenya showing Elgeyo-Marakwet County ***(****http://www.kenyampya.com).*

3.2 Extraction of pyrethrum extracts

Pyrethrum flowers were picked by placing the flower between the index finger and the second finger. Only mature flowers were collected due to increased concentrations of pyrethrins. Approximately 1 Kg of air-dried and ground pyrethrum flowers were extracted by repeated soaking (2 x 48 hours) in 2.5 l mixture of methanol and dichloromethane (1:1 v/v) at room temperature. It was then evaporated to dryness under reduced pressure using a rotary evaporator. This yielded 32.80 g of dichloromethane-methanol crude extract. Vacuum liquid chromatography (VLC) using solvents of increasing polarity i.e hexane, 1:1 v/v hexane/dichloromethane, dichloromethane, 1:1 v/v dichloromethane/ethyl acetate, ethyl acetate, and methanol, was carried out on the dichloromethane-methanol crude extract. This resulted in six VLC fractions which were: VLC 1 (hexane fraction) 3.57 g, VLC 2 (hexane-dichloromethane fraction) 4.42 g, VLC 3 (dichloromethane fraction) 6.25 g, VLC 4 (dichloromethane-ethyl acetate fraction) 4.02 g, VLC 5 (ethyl acetate fraction) 3.75 g and VLC 6 (methanol fraction) 7.75 g. The VLC fractions were preliminarily subjected to *P. aeruginosa*, *MRSA*, *S. sonnie,* and *S. aureus* to determine the most active extract.

3.2.1 Thin-layer chromatography of bio-active dichloromethane VLC fraction

Thin-layer chromatography was performed on plates that had silica GF 254 nm, (Merck, Germany) 0.25 mm thickness. The dichloromethane dry crude extract was first dissolved in dichloromethane followed by spotting on 2×5 aluminum packed TLC plates using a capillary tube. Spotting was done at about half a centimeter from the base on a TLC plate. The plate was then placed in a 100 ml beaker containing 10 ml of the appropriate solvent system then covered with aluminum foil. It was then allowed to develop up to 4 cm up the plate. The developed chromatogram was then visualized under a UV lamp (Uvitec-LF-204.LS) at 254 nm and 365 nm. The solvent mixture that gave optimum separation for the dichloromethane VLC extract was 5:5 acetone: petroleum ether (A: P).

3.2.2 Column chromatography of dichloromethane VLC fraction

A column, 50 cm length and 20 mm diameter, was packed with silica gel 60 0.06-0.2 mm (70-230 mesh ASTM). The dry dichloromethane VLC fraction (6.25 g) was placed on top of silica in the column and the solvent system obtained from TLC analysis was added to the reservoir attached to the column. The column was eluted gradually at approximately 15 ml/5 min and equal volume collected in test tubes. The test tubes with similar TLC patterns were pooled together resulting in 4 fractions. After carrying out bioassay of the column fractions against selected bacteria, fraction 4 was the most active. The amount of fraction 4 obtained was 2.5 g. The fraction was then subjected to preparative high-performance liquid chromatography (HPLC) to obtain pure compounds.

3.2.3 Preparative high-performance liquid chromatography (HPLC) of fraction 4

Fraction 4 of dichloromethane extract was purified using preparative high-performance liquid chromatography equipped with a UV-Vis detector. The fraction was subjected to a reverse phase preparative HPLC using a stationary phase Grom-Sil 120 ODS-5 (250 X 20 mm; 10 µm; Grace Davison, Deerfield, IL, USA) column. The gradient applied was t_0-t_{20} = 5-100% B, t_{21}-t_{23} = 100% B and re-equilibration at 15% (B) until t_{25} with a flow rate of 15 mL/min. Solvents used were water and acetonitrile spiked with 0.1% HCOOH. Three compounds were obtained from fraction 4 and were eluded at different times as follows, compound **1 (**0.028 g)**,** compound **2** (0.6 g), and compound **3** (0.3 g). The compounds were then divided into two portions, one portion of each compound was used for 1D and 2D high field NMR spectroscopy while the other portions were subjected to assays against selected bacteria.

3.2.4 Nuclear magnetic resonance (NMR) spectroscopy of the isolated compounds

The ^{1}H, ^{13}C, DEPT, HSQC, COSY, and HMBC NMR spectra were recorded on the Bruker Advance 500 MHz NMR spectrometer at the Technical University of Berlin, Germany. The readings were done in DMSO and chemical shifts assigned by comparison with the residue proton and carbon resonance of the solvent. Tetramethylsilane (TMS) was used as an internal standard and chemical shifts were given as δ (ppm). The structures were then simulated using the ACD NMR manager program to obtain the chemical shifts of protons. The off-diagonal elements were used to identify the spin-spin coupling interactions in the ^{1}H-^{1}H COSY (Correlation spectroscopy). The proton-carbon connectivity, up to three bonds away, was identified using the ^{1}H-^{13}C Heteronuclear Multiple Bond Correlation (HMBC) spectra. The ^{1}H-^{13}C Heteronuclear Single Quantum Coherence (HSQC) spectrums were used to determine the connectivity of hydrogen to their respective carbon atoms.

3.3 Bioassay of VLC dichloromethane extract, column fractions, and isolated compounds against selected bacteria

Four bacterial strains *S. aureus* (ATCC 25923) *P. aeruginosa* (ATCC 27853), *S. sonnei* (ATCC 25931), and *MRSA* (Clinical isolate) were used for the assay of antibacterial activities. The four were obtained from Kenya Medical Research Institute (KEMRI). The disc diffusion method for antibacterial susceptibility testing was carried out according to NCCLS, (2000). Mueller-Hinton agar was prepared according to the manufacturer's instruction and dispensed at 20 ml per plate in petri dishes. Suspension of selected micro-organism was made in sterile normal saline and adjusted to 0.5 Mc Farland standards. Each labeled medium plate was then inoculated with *P. aeruginosa, S. sonnie, S. aureus,* and *MRSA* by streaking in a form that lawn growth can be observed. Dichloromethane crude extract, column fractions, and isolated pure compounds equivalent to 100 mg/ml, were applied to sterile paper discs (6 mm diameter), and the discs deposited on the surface of the inoculated agar plates. They were then incubated for 24 hrs at 37°C. Mixtures of the isolated compounds in a ratio of (1:1:1) at the same concentration of 100 mg/ml were also subjected to the bioassay as per (Islam *et al.,* 2015).

Zones of inhibition were measured in millimeters after 24 hrs of growth. The inhibition zones less than 10 mm in diameter were not considered for the antibacterial MIC and MBC analysis. For each extract, 3 replicates were assayed. The negative control used in this experiment was 1% dimethyl sulfoxide (DMSO) whereas 30 µg/disc chloramphenicol discs were used as the positive control. The MIC was determined using the microdilution method as described by the Clinical and Laboratory Standards Institute (2009). It was carried out on the extracts that caused inhibition zones ≥10 mm±SD. These extracts were fraction 1, 3, and 4 subjected to *MRSA* and fraction 1, 2, 3, 4 as well as isolated compounds as a mixture (1:1:1) subjected to *P. aeruginosa*. Exactly, 20 µl of the selected fractions and compounds were loaded onto a 96-well titer plate and serially diluted using nutrient broth obtaining a concentration range from 100 to 6.5 mg/ml. Serial dilution was also carried out on the positive control (chloramphenicol).

The dilutions of extracts and those of positive control were placed in two rows of the microtiter plate. The first row served as the test and it had the microbial suspension (5µl) added while the second row served as the control with no micro-organisms added. After incubation for 24 hrs at 37^{o}C, the samples were observed. MIC was recorded as the lowest concentration of each extract that inhibited the bacterial growth as detected by the absence of visual turbidity in comparison with the control. The Minimum Bactericidal Concentration (MBC) assay was adapted from (Sánchez *et al.,* 2016). An aliquot of each well in the MIC 96 well plate was swabbed on the entire surface of Muller Hinton Agar plates and then incubated under the growth conditions which are 37^{o}C for 24 hours. The lowest concentration that prevented the bacterial growth was registered as MBC.

3.4 Green synthesis of silver nanoparticles using different pyrethrum crude extracts

3.4.1 Preparation of different pyrethrum crude extracts

Each twenty grams of powdered flower material was each extracted with 100 ml of different organic solvents at room temperature and evaporated to dryness under reduced pressure. These solvents were hexane, dichloromethane-hexane (1:1 v/v), dichloromethane, dichloromethane-ethyl acetate (1:1v/v), ethyl acetate, dichloromethane-methanol (1:1v/v), and methanol. The aqueous crude extract was also prepared by dissolving 20 g of the flower material in 100 ml of water followed by freeze-drying. The extracts were then used for the green synthesis of silver nanoparticles**.**

3.4.2 Phytochemical analysis of the pyrethrum crude extracts used in the synthesis of Ag NPs

The phytochemical analysis of the organic and aqueous extract was done qualitatively, using standard procedures according to (Balamurugan *et al.,* 2019). The analyzed phytochemicals were tannins, saponins, flavonoids, alkaloids, phenols, glycosides, and terpenoids.

3.4.3 Preparation of silver nanoparticles

Synthesis of silver nanoparticles (Ag NPs) was done according to (De Soyza *et al.,* 2017). Briefly, 0.05% of each organic and aqueous crude extract was prepared. This was followed by mixing 1 mM silver nitrate (Ag NO_3) with the same volume of 0.05% of different extracts and the resultant mixture incubated at 30°C in the dark on a linear shaker. To monitor the completion of bioreduction of Ag^+ to Ag^0 (Silver nanoparticles), samples (1 ml) of the mixture of extracts and Ag NO_3 were periodically collected and diluted with deionized water (10 times). The diluted samples were then scanned using a UV–visible (UV-vis) spectroscopy for a prominent peak at around 410-460 nm. The scanning was done between 300 nm and 700 nm using (Agilent technologies Cary 60 UV-vis). The UV-vis spectra of the extracts and Ag NO_3 solution were also recorded. Observing a color change of the mixture to brown also indicated the formation of silver nanoparticles (Ag NPs). Once the nanoparticles had formed, the nano-preparation (a mixture of the extracts and Ag NO_3), was then centrifuged at 5000 rpm for 10 minutes. The supernatant was discarded and the pellet containing Ag NPs was then washed and air-dried in an incubator.

3.4.4 Characterization of silver nanoparticles

a) Scanning electron microscopic (SEM) analysis / Energy dispersive X-ray (EDX) analysis

The morphological characteristics of the synthesized silver nanoparticles were determined using a scanning electron microscope (SEM Carl Zeiss Ultra Plus). The samples were first converted to a dry powder then gold-coated using a sputter coater and mounted on a sample handler. It was then followed by scanning the sample using a focused beam of electrons. The details pertaining applied voltage, magnification used, and size of the contents of the images were implanted on the images themselves. The elemental analysis of the nanoparticles was carried through spectrum and elemental mapping using energy dispersive X-ray (EDX) analyzer (Oxford X max) attached to the scanning electron microscope after the nanoparticles had been dehydrated and covered using a carbon layer

b) Transmission electron microscopic (TEM) analysis

A drop of synthesized Ag NPs was placed on the carbon-coated copper grids and kept overnight under vacuum desiccation before loading them onto a specimen holder. TEM micrographs of the sample were taken using the (TEM JEOL, JEM 1010) instrument operated at an accelerating voltage of 200 kV.

c) Fourier transform infrared spectroscopy (FTIR) analysis

The analysis of dried Ag NPs was carried out through the potassium bromide (KBr) pellet (FTIR grade) in a 1:100 ratio. The samples were scanned using infrared in the range of 4000–400 cm^{-1} using Fourier Transform Infrared Spectrometer (FTIR-600 Spectrometer). The spectral data obtained were compared with the reference chart to identify the functional groups present in the sample.

3.4.5 Bioassay of green synthesized Ag NPs

It was done as described in section 3.3 with variation in concentrations used. Sterile paper discs 6 mm in diameter each containing about 500 µg/ml of Ag NPs, plant extracts (control), and Ag NO_3 (control), were deposited on the incoculated agar plates and zone of inhibition determined. For MIC determination, serial dilutions of dichloromethane-Ag NPs and dichloromethane-methanol-Ag NPs were prepared in a 96-well microtiterplate, obtaining a concentration range from 500 µg/ml to 15.625 µg/ml. MIC was recorded as the lowest concentration of Ag NPs that inhibited the bacterial growth as detected by absence of turbidity.

3.5 Cytotoxicity of pyrethrum extracts and biosynthesized Ag NPs

Pyrethrum crude extracts used in the synthesis of Ag NPs, synthesized Ag NPs, and pure compounds as a mixture (1:1:1) were tested for in vitro cytotoxicity against Vero cells using MTT colorimetric assay (Mosmann, 1983). This is because these extracts had shown bioactivity against the selected micro-organisms. These cells were acquired from KEMRI Nairobi. The Cells were first grown in Minimum Essential Medium (MEM) Eagle's Base supplemented with 15% Fetal Bovine Serum (FBS), 2.62 g/L $NaHCO_3$, 20 mM L-glutamine, 10ml/L Penstrep 0.5 mg, and Fungizoid using a T-75 culture flask for 48 hours.

After 48 hours, the Phosphate Buffered Saline (PBS) was used to wash the cells in the flask, and trypsin was used to detach the cells. An aliquot of 2.0×10^5 cells/ml suspension was then seeded in a 96-well plate. It was then incubated at temperatures of 37^oC for 24 hours at 5% CO_2. This allowed the cells to attach to the plate.

Briefly, 150 µl of 100 µg/ml of various extracts i.e (Pyrethrum crude extracts used in the synthesis of Ag NPs, synthesized Ag NPs, and pure compounds as a mixture (1:1:1 v/v) were added to row H. Serial dilutions were carried out by pipetting 50 µl from wells of row H and adding to wells of row G. Another 50 µl was then transferred from row G to wells of row F. The same procedure was carried out to row B and finally discarding the last 50 µl of this row. It resulted in three-fold serial dilutions from row H to row B. Row A acted as the cell control (cells without extract treatment). Medium control (blank medium) was also incorporated in the same plates. The plates were then incubated for 48 hours at 37°C and 5% CO_2.

After 48 hours, MTT was added and incubated for 2 hours at 37°C and 5% CO_2. The media in the 96- well plate was discarded. Fifty microliters of DMSO were kept in all the plates and shaken to dissolve the formazan crystals. The plate was then put in an ELISA reader and the absorbance read between 540 nm and 720 nm. The percentage of cell viability was then calculated using the formula

$$\%\ Cell\ Viability = (AT - AB)/(AC - AB) \times 100$$ (Nemati *et al.*, 2013)

Where AT = Absorbance value of test compound.

AB =Absorbance value of the blank

AC= Absorbance value of the control

3.6 Data analysis

The comparison of means of the zone of inhibition was done using one-way ANOVA. The differences were significant at ($P \leq 0.05$).

CHAPTER FOUR

RESULTS

4.1 Preliminary screening of pyrethrum VLC extracts

Preliminary screening of *C. cinerariaefolium* crude extracts against selected bacteria is shown in Table 1. Appendix 1 shows the larval bioassay results. The concentration used for preliminary screening was 100 mg/ml. The diameter of the zone of inhibition by the extracts on the selected bacteria was determined in millimeters (mm) and obtained as mean±SD. Dichloromethane VLC extract showed the highest zone of inhibition against the selected bacteria. The observed zone of inhibitions were 10.7±1.2, 10.3±0.6, 7.2±0.3, and 6.3±0.3 mm against *MRSA, S. aureus, P. aeruginosa, S. sonnie* respectively, hence the dichloromethane extract was selected for further fractionation to isolate bioactive compounds.

Table 1: Preliminary bioassay results of VLC extracts against selected bacteria at 100 mg/mL

	Test organisms			
Extracts	***MRSA***	***S. aureus***	***P. aeruginosa***	***S. sonnie***
Hexane	6±0	8.3±0.6	6±0	6±0
Dichloromethane-hexane	8.7±1.2	8.3±1.2	6±0	6±0
Dichloromethane	10.7±1.2	10.3±0.6	7.2±0.3	6.3±0.3
Dichloromethane-ethyl acetate	9.7±0.6	7.7±1.2	6±0	6±0
Ethyl acetate	6±0	6±0	6±0	6±0
Methanol	6±0	6±0	6±0	6±0
Dichloromethane-methanol	7.2±0.3	6.7± 0.2	6.3±0.3	6±0
Chloramphenicol ^{P}	26.7±1.2	24.2±0.8	26±1	24.7±1.3
DMSO+distilled H_2O ^{Q}	6±0	6±0	6±0	6±0

^{P} Positive control, ^{Q} negative control

4.2 Characterization and identification of bioactive compounds isolated from dichloromethane fraction of *C. cinerariaefolium*

The three isolated compounds i.e compounds **1, 2,** and **3** subjected to 1D and 2D NMR analysis resulted in various spectral data belonging to ^{1}HNMR, ^{13}CNMR, DEPT, COSY, HMBC, and HSQC that were used to carry out structure elucidation. The isolated compounds were oily with compounds **1** and **2** being colorless while compound **3** was pale yellow.

4.2.1 Structure elucidation of jasmolin I

A total of 21 carbons were observed in compound **1** a**s** illustrated in Table 2, on analyzing the spectra for ^{13}CNMR (appendix 2), DEPT (appendix 3) and HSQC (appendix 4). These carbons were attributable to a carbonyl carbon at δ_C 203.1 (C-4′), an ester carbonyl carbon at δ_C 171.4 (C-4), four quaternary carbons at δ_C 164.8 (C-2′), δ_C 141.5 (C-3′), δ_C 134.5 (C-8) and δ_C 28.2 (C-2), six methine carbons at 132.4 (C-9′), δ_C 124.3 (C-8′), δ_C 120.9 (C-7), δ_C 72.9 (C-1′), δ_C 33.6 (C-1) and δ_C 32.2 (C-3), six methylene carbons at δ_C 41.5 (C-5′), δ_C 21.6 (C-10′) and δ_C 20.7 (C-7′), and six methyl carbons at δ_C 25.3 (C-10), δ_C 20.1 (C-6), δ_C 20.0 (C-5), δ_C 18.1 (C-9), δ_C 13.9 (C-11′), and δ_C 13.7 (C-6′).

The HMBC spectrum (appendix 5) showed the correlation between carbons and protons that are two to three bonds away. From the HMBC spectrum, cross-peaks from H-5 and H-6 methyl protons to C-1 and C-2 suggested the location of the methyl groups at C-2 while the attachment of the 2-methylprop-1-en-1-yl group to C-3 was suggested by the HMBC cross-peaks from H-7 to C-1. The pent-2-en-1-yl group was determined to be at C-2′ based on the HMBC cross-peaks from H-7′ to C-2′, C-3′, C-4′ and those from H-8′ to C-3′. The protons for the two methyl groups attached to the cyclopropane ring resonated at δ_H 1.22 (3H, s) and 1.12 (3H, s). Additionally, the ^{1}HNMR spectrum (appendix 6) showed another signal at δ_H 5.65 (1H, dd, J = 18.6, 1.7 Hz), 2.82 (1H, dd, J = 18.6, 6.3 Hz) and δ_H 2.13 (1H, dd, J = 6.3, 1.7 Hz) corresponding to the cyclopentenone ring protons H-1′, H-5a′ and H-5b′, respectively.

The COSY spectra (Appendix 7) showed a correlation between H-3 and H-1, H-1′ and H-5′, H-7′ and H-8′, H-9′ and H-10′, H-11′ and H-12′. The HMBC and COSY correlations for the compound is illustrated in figure 15. From the evidences presented in Table 2 and previous studies the structure of compound **1** was elucidated as (Z)-2-methyl-4-oxo-3-(pent-2-en-1-yl)cyclopent-2-en-1-yl2,2-dimethyl-3-(2-methylprop-1-en-1-yl)cyclopropane-1-carboxylate commonly known as Jasmolin I (Hata *et al.*, 2011). The structure of compound **1** is presented in figure 16.

Table 2: The assignment of ^{13}CNMR, ^{1}HNMR, DEPT and HMBC of jasmolin I

Position	δ^{1}HNMR, (*J* in Hz)	δ ^{13}CNMR	DEPT	HMBC	LITERATURE δ^{1}HNMR *
1	1.55, d (5.3)	33.6,	CH	C-2, C-3, C-4, C-5,	1.32

				C-6, C-7	
2		28.2,	Cq		
3	1.96, dd (8.1, 5.3)	32.2,	CH	C-1, C-2, C-4, C-8	2.00
4		171.4,	Cq		
5	1.12, s	20.0,	CH_3	C-1, C-2, C-6	1.05
6	1.22, s	20.1,	CH_3	C-1, C-2, C-5	1.18
7	4.98, dq (8.1, 1.4)	120.9,	CH	C-1, C-9, C-10	4.84
8		134.5,	Cq		
9	1.68, d (1.3)	18.1,	CH_3	C-7, C-8, C-10	1.63
10	1.71, d (1.4)	25.3,	CH_3	C-7, C-8, C-9	1.62
1′	5.65, dd (18.6, 1.7)	72.9,	CH	C-4, C-2′, C-3′, C-4′	5.58
2′		164.8,	Cq		
3′		141.5,	Cq		
4′		203.1,	Cq		
5a′	2.13, dd (6.3, 1.7)	41.5,	CH_2	C-2′, C-3′, C-4′	2.13
5b′	2.82, dd (18.6, 6.3)			C-1′, C-4′, C-6′	2.75
6′	2.03, s	13.7,	CH_3	C-1′, C-2′, C-3′	1.95
7′	2.92, dd (7.3, 1.7)	20.7,	CH_2	C-2′, C-3′, C-4′, C-8′, C-9′	2.89
8′	5.22, dt (10.5, 7.4)	124.3,	CH	C-3′, C-7′, C-9′	5.33
9′	5.39, dt (10.5, 1.7)	132.4,	CH	C-8′, C-10′	5.16
10′	2.15, m	21.6,	CH_2	C-8′, C-9′	2.03
11′	0.96, t (7.5)	13.9,	CH_3	C-9′	0.90

*Hata *et al.*, 2011

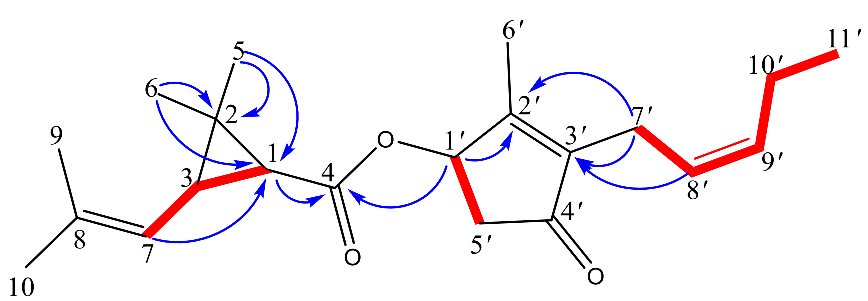

Figure 15: HMBC (blue) and COSY (red bold lines) correlations of Jasmolin I

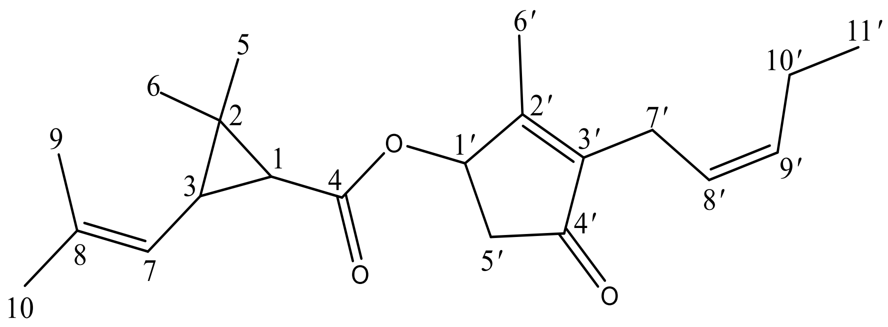

Figure 16: (Z)-2-methyl-4-oxo-3-(pent-2-en-1-yl) cyclopent-2-en-l-yl 2, 2-dimethly-3-(2-methylprop-1--en-1-yl) cyclopropane-1-carboxylate (Jasmolin I) (compound 1)

4.2.2 Structure elucidation of Pyrethrin II

The ^{1}HNMR (appendix 8), ^{13}CNMR (appendix 9), and DEPT (appendix 10) spectra for compound **2**, closely resembled those of compound **1** except for a few noted differences. First, there was a disappearance of the C-9 methyl carbon signal accompanied by the appearance of methyl ester carbon signals at δ_C 167.6 (C-9) and δ_C 52.1 (9-OCH_3) attached to C-8 in compound **2**. The attachment of this group at C-8 was suggested by the HMBC cross-peaks from the olefinic proton H-7 to the ester carbonyl carbon at C-9 (appendix 11). Secondly, there was the replacement of the pent-2-en-1-yl substituent in compound **1** with the penta-2,4-dien-1-yl substituent in 2 as evident from the doublet at δ_H 3.10 (2H, d, J = 7.7, H-7′), a doublet of triplet at δ_H 6.85 (1H, dt, J = 16.8, 10.8, H-8′), a triplet at δ_H 6.01 (1H, t, J = 10.8, H-9′), a multiplet at δ_H 5.36 (1H, m, H-10′) and a set of two doublet of doublets for the two-terminal olefinic protons at δ_H 5.27 (1H, dd, J = 16.8, 2.2, H-11a′) and δ_H 5.20 (1H, dd, J = 10.2, 2.2, H-11b′).

The direct bonding of proton to carbons was derived from the HSQC spectra (appendix 12) while the COSY spectra gave the correlation between H-3 and H-1, H-1′ and H-5′, H-7′ and H-8′, H-9′ and H-10′, H-11′, and H-12′ as in jasmolin I. Based on the spectral information (Table 3) and literature comparison (Rugutt *et al.*, 1999), compound **2** was elucidated to be 2-methyl-4-oxo-3-(Z)-penta-2,4-dien-1-yl)cyclopent-2-en-1-yl-3-((E)-3-methoxy-2-methyl-3-oxoprop-1-en-1-yl)-2,2-dimethylcyclopropane-1-carboxylate, commonly known as Pyrethrin II. The correlations for HMBC and COSY for compound **2** is presented in figure 17 while the structure for the compound is presented in 18.

Table 3: **The assignment of ^{1}H NMR, ^{13}C NMR, DEPT, and HMBC of pyrethrin II**

No.	δ_H NMR, (*J* in Hz)	δ_C NMR	DEPT	HMBC	LIT δ_C, Type
1	2.08, d (5.2)	35.2	CH	C-2, C-3, C-5, C-6, C-7	34.5, CH
2		30.4	Cq		29.0, C
3	2.15, dd (9.7, 5.2)	32.6	CH	C-1, C-2, C-4, C-8	32.9, CH
4		171.0	Cq		172.2, C
5	1.20, s	22.1	CH_3	C-1, C-2, C-6	22.0, CH_3
6	1.26, s	20.7	CH_3	C-1, C-2, C-5	20.3, CH_3
7	6.50, dt (9.7, 1.4)	139.8	CH	C-1, C-9, C-10	
8		129.1	Cq		143.0, C
9		167.6	Cq	C-7, C-8, C-10	
10	1.89, d (1.4)	13.1	CH_3	C-7, C-8, C-9	
1′	5.67, dd (18.5, 1.7)	73.7	CH	C-4, C-2′, C-3′, C-4′	73.4, CH
2′		166.6	Cq		164.9, C
3′		141.5	Cq		142.2, C
4′		203.5	Cq		203.5,C
5a′ 5b′	2.15, dd (6.3, 1.7) 2.85, dd (18.5, 6.3)	41.9	CH_2	C-2′, C-4′ C-4, C-1′, C-4′, C-6′	41.9, CH_2
6′	2.04, s	14.2	CH_3	C-1′, C-2′, C-3′, C-4′	14.3, CH_3
7′	3.10, d (7.7)	22.1	CH_2	C-2′, C-3′, C-4′, C-8′, C-9′	21.9, CH_2
8′	6.85, dt (16.8, 10.8)	132.4	CH	C-9′, C-10′	126.7, CH
9′	6.01, t	130.5	CH	C-7′	130.4, CH

	(10.8)				
10′	5.36, m	127.8	CH	C-8′	131.5, CH
11a′	5.27, dd	118.8,	CH_2	C-9′	
11b′	(16.8, 2.2)			C-9′	118.4,CH_2
	5.20, dd				
	(10.2, 2.2)				
9-OCH_3	3.68, s	52.1	CH_3	C-9	51.8, 0CH_3

***** (Rugutt *et al.*, 1999)

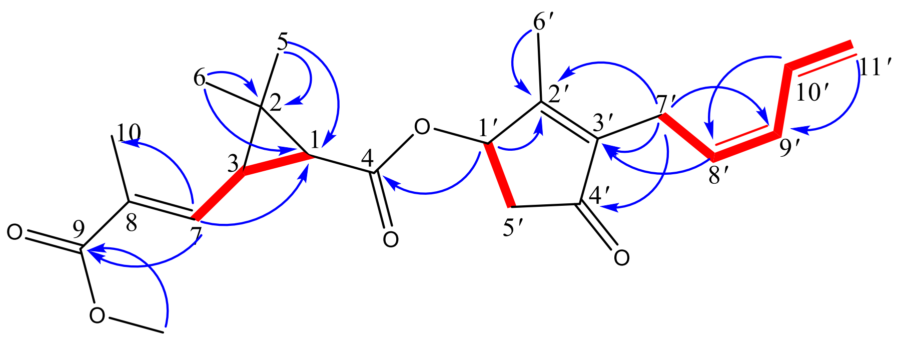

Figure 17: HMBC (blue) and COSY (red bold lines) correlations of Pyrethin II

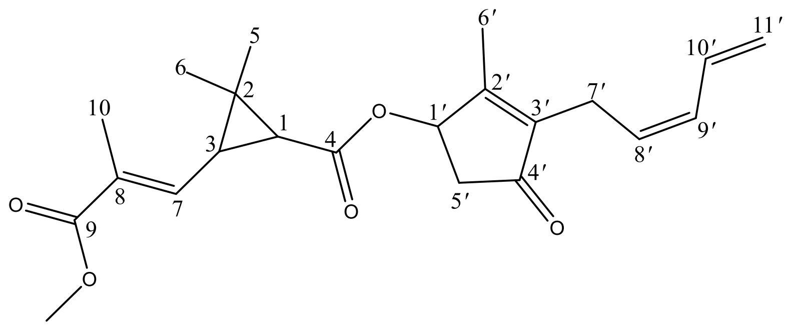

Figure 18: 2-methly-4-oxo-3(Z)-penta-2,4-diene-1-yl) cyclopent-2-en-1-yl-3-(E)-3-methoxy-2-methly-3-oxoprop-1-en-yl-2,2-dimethlycyclopropane-1-carboxylate (Pyrethrin II) (compound 2)

4.2.3 Structure elucidation of cinerolone

The ^{1}HNMR spectrum (appendix 13) displayed signals for a doublet due to the exocyclic methylene protons at $^\delta{}_H$ 2.90 (2H, d, J = 6.2, H-1'), two sets of multiplets for the olefinic protons at $^\delta{}_H$ 5.27 (1H, m, H-2') and $^\delta{}_H$ 5.27 (1H, m, H-3'), and a singlet at $^\delta{}_H$ 1.03 (3H, s, H-4') for the but-2-en-1-yl substituent. A signal for methyl protons was also observed at $^\delta{}_H$ 2.01 (3H, s, H-6).

The ^{13}CNMR (appendix 14), DEPT (appendix 15), and HSQC (appendix 16) data for compound **3** exhibited 10 carbon signals which were classified as carbonyl carbon (δ_C 203.1, C-1), two quaternary carbons at δ_C 170.9 (C-3) and δ_C 138.2 (C-2), three methine carbons at δ_C 131.9 (C-3′), δ_C 126.8 (C-2′) and δ_C 69.9 (C-4), two methylene carbons at δ_C 43.9 (C-5) and δ_C 21.4 (C-1′), and two methyl carbons at δ_C 22.8 (C-4′) and δ_C 13.6 (C-6). The HMBC cross-peaks from H-5 to C-1/C-3/C-4 and from H-4 to C-2/C-3 revealed that the carbonyl group was at C-1 and the hydroxyl group was attached to C-4. The HMBC (appendix 17) cross-peaks from H-1′ to C-1′/C-2′/C-3′ were used to locate the but-2-en-1-yl substituent at C-2, while the HMBC cross-peaks from the methyl protons H-6 to C-2/C-3 suggested the attachment of this methyl group at C-3. COSY spectra (appendix 18) showed correlation between 4-H and 5-H, while1'-H correlated with 2'-H and 2'-H correlated with 3'-H. The compound was therefore characterized as (Z)-2-(but-2-en-1-yl)-4-hydroxy-3-methylcyclopent-2-en-1-one, commonly known as Cinerolone based on spectral data in Table 4 and literature findings (Bramwel *et al.*, 1969). The HMBC, and COSY correlations for compound **3** are shown in figure 19 while the structure for the compound is presented in figure 20.

Table 4: The assignment of ^{1}H NMR, ^{13}C NMR, DEPT, and HMBC of cinerolone

Position	δ^1HNMR, (*J* in Hz)	δ^{13}CNMR	DEPT	HMBC	LITERATURE HNMR*
1		204.8	C=O		
2		138.2	C		
3		170.9	C		
4	4.52, d (6.3)	69.9	CH	C-2, C-3	5.31
5a	2.63, dd (18.1, 6.3)	43.9	CH_2	C-1, C-3, C-4	7.76
5b	2.06, m				7.22
6	2.01, s	13.6	CH_3	C-1, C-2, C-3	7.9
1′	2.90, d (6.2)	21.4	CH_2	C-1, C-2, C-3, C-2′, C-3′	7.06
2′	5.27, m	126.8	CH	C-1′	
3′	5.27, m	131.9	CH		
4′	1.03, s	22.8	CH_3		

4-OH	8.15, s

*(Bramwell *et al.*, 1969)

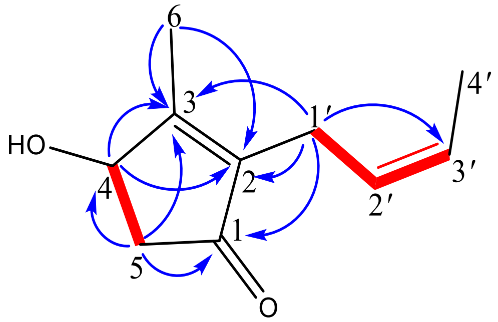

Figure 19: HMBC (blue) and COSY (red bold lines) correlations of cinerolone

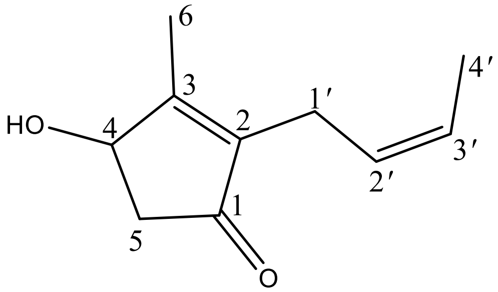

Figure 20: (Z)-2-(but-2-en-1-yl)-4-hydroxy-3-methylcyclopent-2-en-1-one (cinerolone) (compound 3)

4.3 Antibacterial activity of dichloromethane column fractions and isolated compounds from *C. cinerariaefolium*

4.3.1 Disc diffusion assay

Results of the bioassay against the selected organisms using disc diffusion assay are as shown in Table 5. The values are the mean of three experiments ± S.D. Within a column, the inhibition zones of extracts sharing the same letters were not significantly different while those with different letters were significantly different ($\alpha = 0.05$, one-way ANOVA). From the results, fraction 4 was the most active fraction against *MRSA*, *S. aureus* and *P. aeruginosa* while non of the fractions were active on *S. sonnie*. Individaully the isolated compounds were not active on the selected bacteria except compound 1. When mixed, the isolated showed significant activity against the selected bacteria. The images of bioassay of isolated compounds as a mixture (1:1:1) against *P. aeruginosa* and *S. aureus* are in figure 21 while *MRSA* and *S. sonnie* images are in figures 22. Appendix 19 shows the bacterial bioassay results.

Table 5: Inhibition zones of column fractions and isolated compounds on the test organisms at 100 mg/mL

Extracts	**Zone of inhibition in mm**			
	MRSA	***S. aureus***	***P. aeruginosa***	***S. sonnie***
Fraction 1	12±0.5[a]	7±0.5[b]	11.7±0.6[b]	6±0[b]
Fraction 2	7.6±0.6[b]	7.4±0.6[bg]	11.6±0.5[b]	6±0[b]
Fraction 3	11±1[a]	6.6±0.2[b]	17.3±1.2[c]	6±0[b]
Fraction 4	12.3±0.6[a]	9.8±1[a]	22.7±1.2[d]	6±0[b]
Compound 1	6±0[e]	6±0[f]	7.7±0.6[a]	6±0[b]
Compound 2	6±0[e]	6±0[f]	6±0[e]	6±0[b]
Compound 3	6±0[e]	6±0[f]	6±0[e]	6±0[b]
Compound mixture (1:1:1 v/v)	7.3±0.6[b]	8.2±0.3[g]	14±0[g]	6±0[b]
Chloramphenicol [P]	26.7±1.2[c]	24.2±0.8[c]	26±1[f]	24.7±1.3[c]
Dmso+distilled H_2O[Q]	6±0[e]	6±0[f]	6±0[e]	6±0[b]

* Within a column similar letters show no significant differences while different letters show a significant difference. [P] Positive control, [Q] Negative control

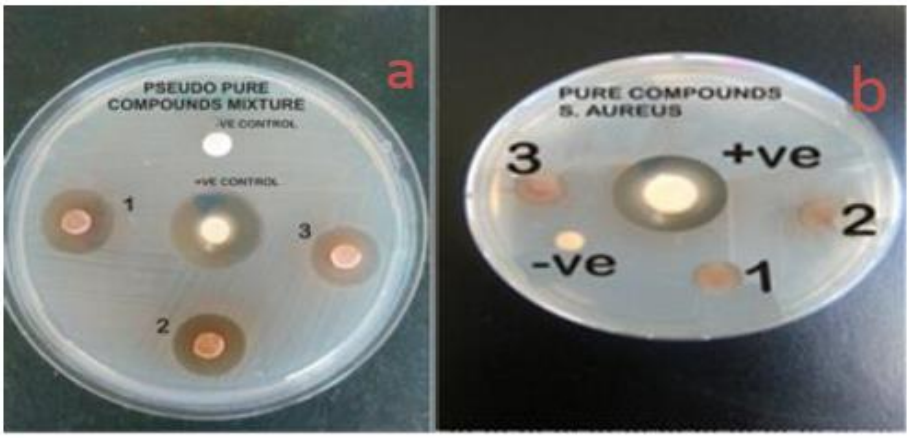

Figure 21: Screening of isolated compounds as a mixture in triplicate at a concentration of 100 mg/ml against *P. aeruginosa* (a) and *S. aureus* (b)

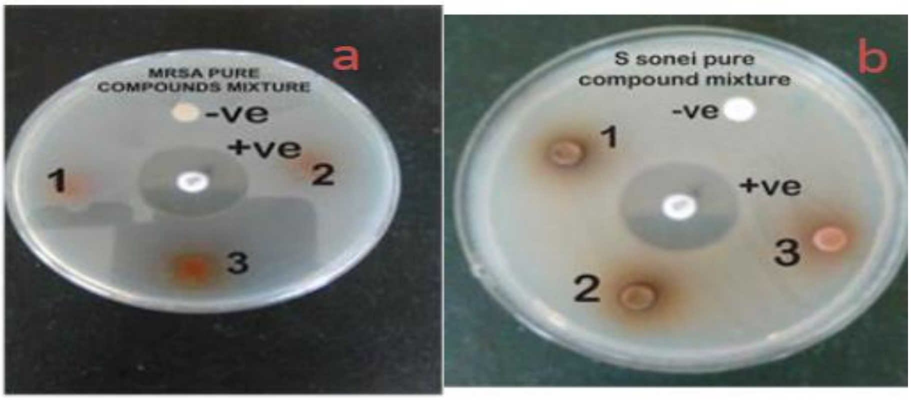

Figure 22: Screening of isolated compounds as a mixture in triplicate at a concentration of 100 mg/ml against MRSA (a) and S. sonnie (b)

4.3.2 Minimum inhibitory concentration (MIC) and minimum bacteriostatic concentration (MBC)

The extracts subjected to MIC and MBC assay were fraction 1, 3, and 4 against *MRSA* and fraction 1, 2, 3, 4 and isolated compounds as a mixture (1:1:1) against *P. aeruginosa.* Serial dilutions of the extracts was done in a 96 well plate to ascertain the MICs for fractions 1, 3, and 4 against *MRSA*, fractions 1, 2, 3, and 4 against *P. aeruginosa*, and isolated compounds as a mixture (1:1:1) subjected to *P. aeruginosa*. From the turbidity observation made on the growth of bacteria in the 96 well plates, the MICs for fraction 1, 3, and 4 against *MRSA*, were 12.5 mg/mL, 12.5 mg/mL, and 6.5 mg/mL, respectively. The MIC for fraction 1, 2, 3, 4 and compound mixture (1:1:1) against *P. aeruginosa* were 25 mg/mL, 25 mg/mL, 25 mg/mL, 12.5 mg/mL, and 25 mg/mL respectively. The MBC for fraction 1, 3 and 4 against *MRSA* were 25 mg/mL, 25 mg/mL, and 12.5 mg/mL respectively. The MBC for fraction 1, 2, 3 and 4 against *P. aeruginosa* were 50 mg/mL 50 mg/mL, 50 mg/mL and 25 mg/mL respectively while for the compound mixture the MBC was 50 mg/mL. For the positive control, the MIC and MBC were 3.125 µg/ml and 6.25 µg/ml respectively against *MRSA* while for *P. aeruginosa* the MIC and MBC were 6.25 µg/ml and 12.5 µg/ml respectively.

4.4 Synthesis and characterization of Ag NPs

On mixing colorless aqueous Ag NO_3 (figure 23) with different crude extracts of *C. cinerariaefolium* i.e dichloromethane extract figure 24 (a), dichloromethane-methanol extract figure 25 (a), dichloromethane-ethyl acetate extract figure 26 (a), methanol extract figure 27 (a), ethyl acetate extract figure 28 (a), aqueous extract figure 29 (a), hexane extract figure 30 (a), and dichloromethane-hexane extract figure 31 (a), followed by storing the solution in the dark, a colour change of the mixture to brown was observed. The colour change was observed in all the mixture except the mixture that contained hexane extract figure 30 (b) and dichloromethane-hexane extract figure 31 (b). The brown color signified suspension of Ag NPs formed in the mixture because of the reduction of silver ions by the phytochemicals in the crude extracts. Therefore Ag NPs were formed in six extracts, resulting in dichloromethane-Ag NPs figure 24 (b), dichloromethane-methanol-Ag NPs, figure 25 (b), dichloromethane-ethyl acetate-Ag NPs figure 26 (b), methanol-Ag NPs figure 27 (b), ethyl acetate extract-Ag NPs figure 28 (b) and aqueous extract-Ag NPs figure 29 (b).

The duration taken for the colour change varied depending on the crude extract used, as shown in Table 6. The UV-visible (UV-vis) spectra of the mixture further confirmed the synthesis of the Ag NPs. This is because the (UV-vis) spectra showed localized peak between 410-460 nm, an indication of the presence of Ag NPs (Migel, 2017). In dichloromethane-Ag NPs, the peak occurred at 434 nm figure 32 (a), dichloromethane-methanol-Ag NPs at 430 nm figure 33 (a), dichloromethane-ethyl acetate-Ag NPs at 446 nm figure 34 (a), methanol-Ag NPs at 445 nm figure 35 (a), ethyl acetate- Ag NPs at 449 nm figure 36 (a), and aqueous-Ag NPs at 439 nm figure 37 (a). The localized peak was absent in aqueous silver nitrate (figure 38) and all plant extracts used in the synthesis.

The absence of the localized peak between 410-460 nm in the UV-vis spectra of various plant extracts is shown in figure 32 (b) for dichloromethane extract, dichloromethane-methanol extract, figure 33 (b), dichloromethane-ethyl acetate extract, figure 34 (b), methanol extract, figure 35 (b), ethyl acetate extract, figure 36 (b), aqueous extract, figure 37 (b), dichloromethane-hexane extract, figure 39 (a), and hexane extract, figure 40 (a). There was also the absence of the localized peak in the mixture that had Ag NO_3 and dichloromethane-hexane extract, figure 39 (b) and a mixture of Ag NO_3 and hexane extract figure 40 (b). This indicated absence of Ag NPs in the two extracts. Appendix (20-24) shows the absorbance values at various wavelengths for the UV-vis spectra belonging to all plant extracts, synthesized Ag NPs, and aqueous silver nitrate.

Figure 23: Aqueous silver nitrate (Ag NO_3) (1mM)

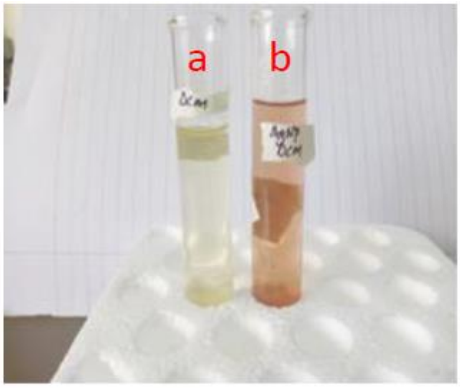

Figure 24: Dichloromethane plant extract (a), a mixture of aqueous Ag NO_3 and dichloromethane extract (dichloromethane-Ag NPs) (b)

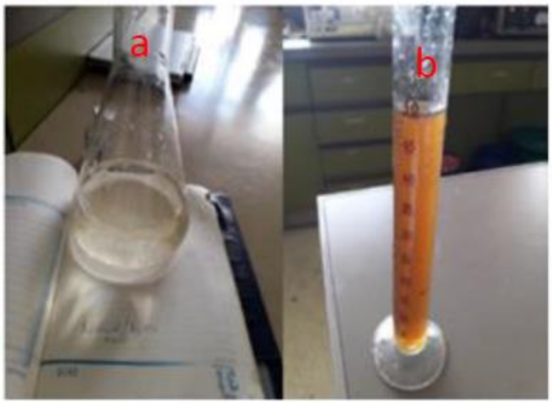

Figure 25: Dichloromethane-methanol plant extract (a), a mixture of Ag NO_3 and dichloromethane-methanol extract (dichloromethane-methanol-Ag NPs) (b)

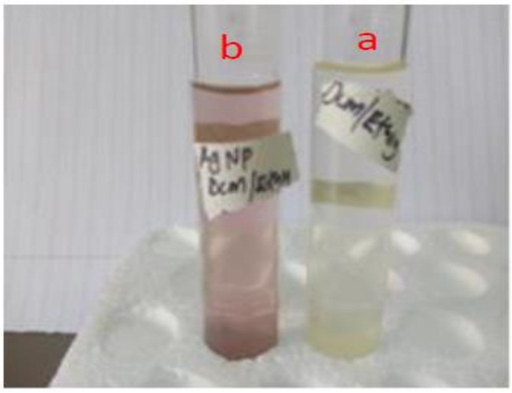

Figure 26: Dichloromethane-ethyl acetate extract (a), a mixture of dichloromethane-ethyl acetate and Ag NO_3 (dichloromethane-ethyl acetate-Ag NPs) (b)

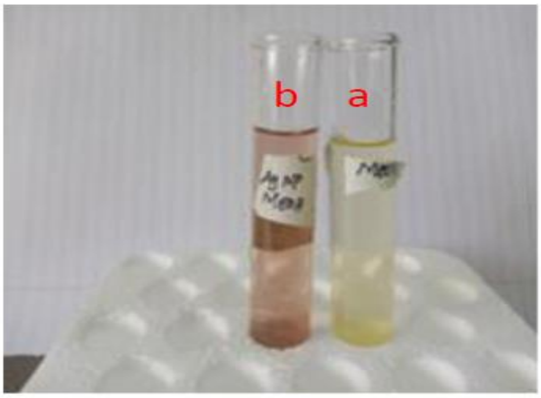

Figure 27: Methanol plant extract (a), a mixture of Ag NO_3 and methanol extract (methanol-Ag NPs) (b)

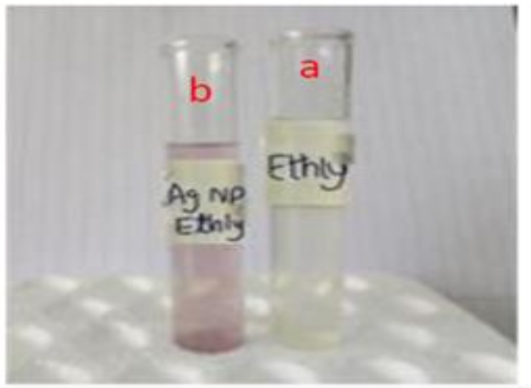

Figure 28: Ethyl acetate extract (a), a mixture of Ag NO_3 and ethyl acetate extract (ethyl acetate-Ag NPs) (b)

Figure 29: Aqueous extract (a), mixture of Ag NO_3 and aqueous extract (aqueous -Ag NPs) (b)

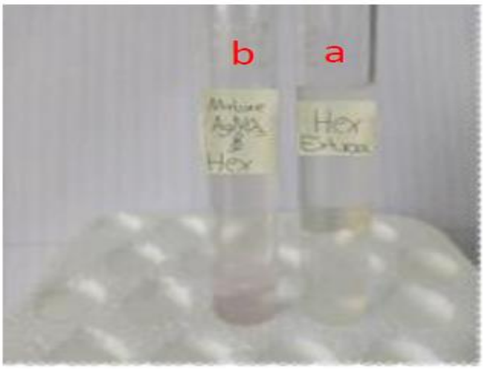

Figure 30: Hexane extract (a), a mixture of Ag NO_3 and hexane extract (b)

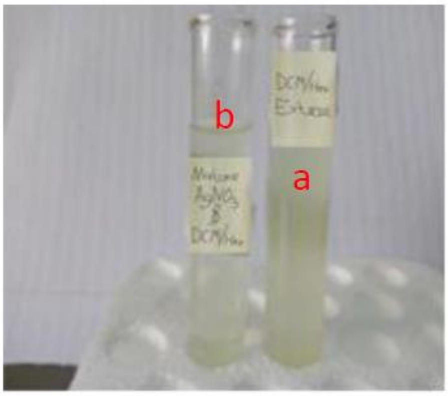

Figure 31: Dichloromethane-hexane extract (a), a mixture of Ag NO_3 and dichloromethane-hexane extract (b)

Table 6: Duration taken for silver nanoparticles to form in different crude extracts

Crude extracts	**Duration**
Dichloromethane	48 hours
Dichloromethane-methanol	48 hours
Dichromethane-ethyl acetate	96 hours
Methanol	48 hours
Ethyl acetate	96 hours
Aqueous	48 hours

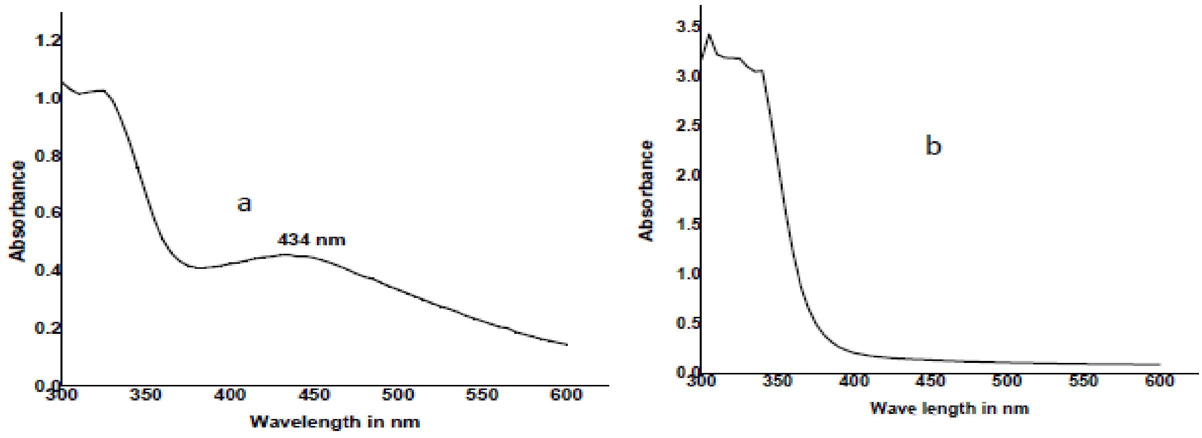

Figure 32: UV-vis spectra of dichloromethane-Ag NPs (a), dichloromethane plant extract (b)

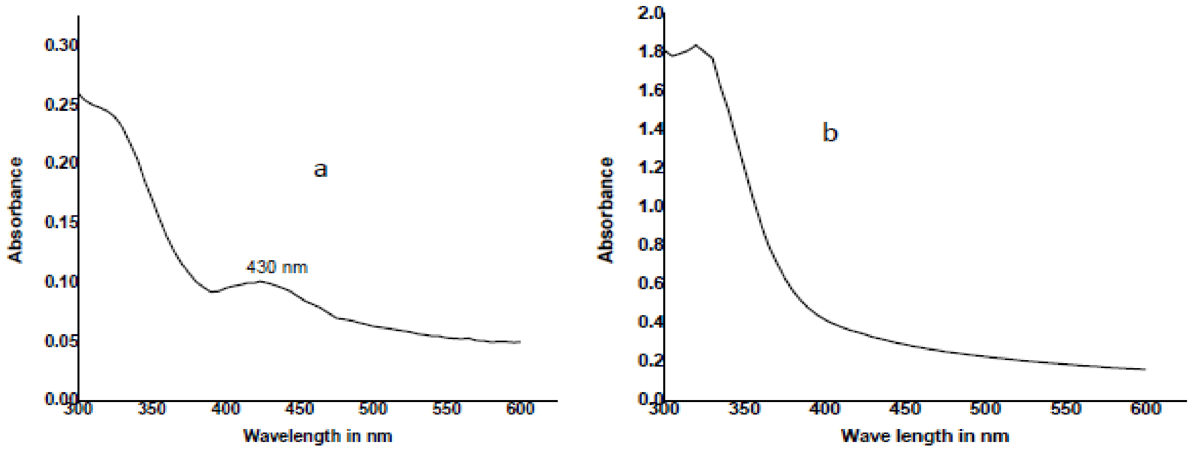

Figure 33: UV-vis spectra of dichloromethane-methanol-Ag NPs (a), dichloromethane-methanol plant extract (b)

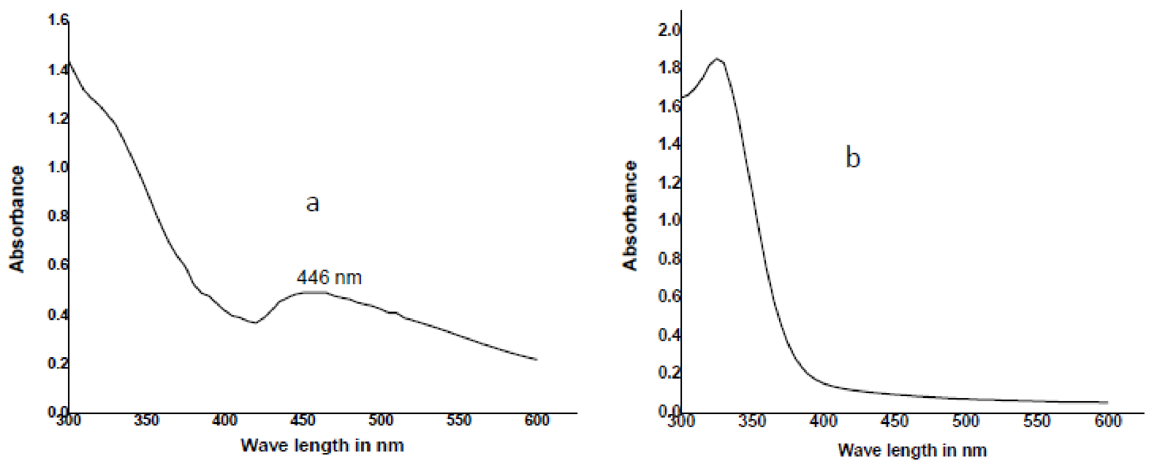

Figure 34: UV-vis spectra of dichloromethane-ethyl acetate-Ag NPs (a), dichloromethane-ethyl acetate plant extract (b)

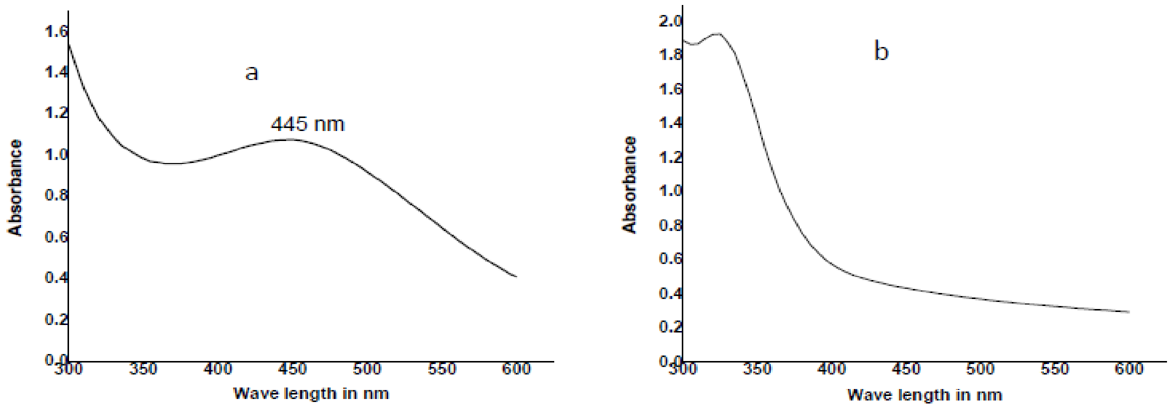

Figure 35: UV-vis spectra of methanol-Ag NPs (a), methanol plant extract (b)

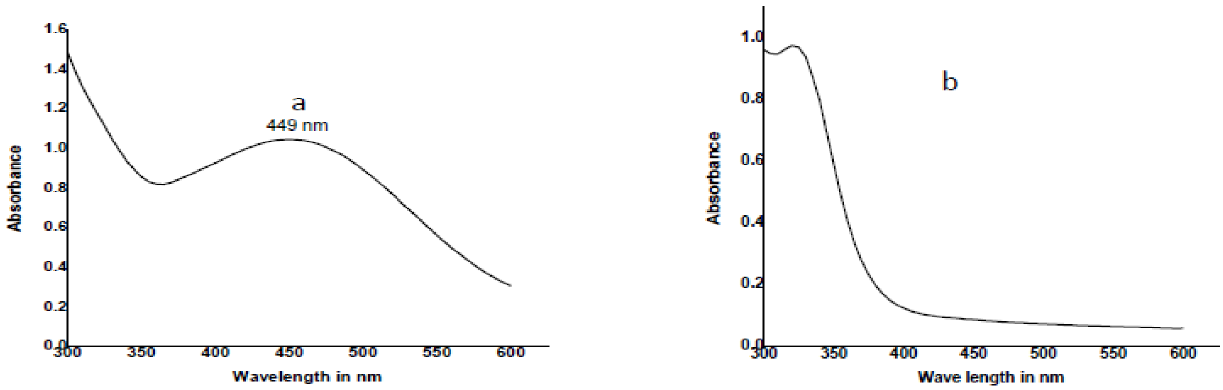

Figure 36: UV-vis spectra of ethyl acetate -Ag NPs (a), ethyl acetate plant extract (b)

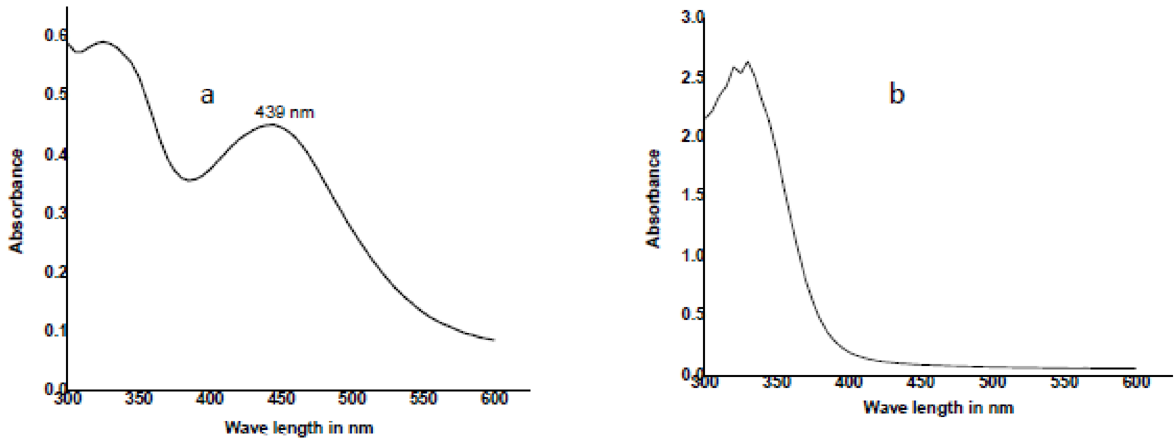

Figure 37: UV-vis spectra of aqueous -Ag NPs (a), aqueous plant extract (b¿)

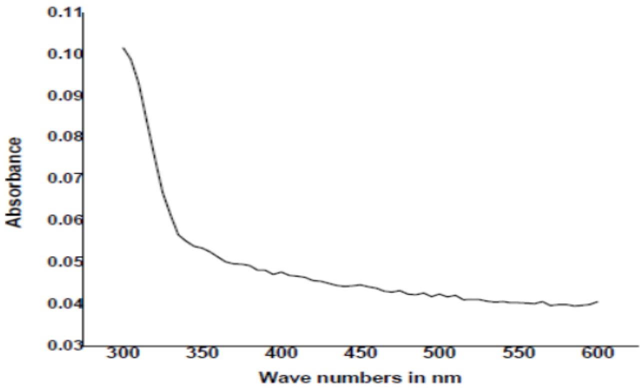

Figure 38: UV-vis spectra of silver nitrate

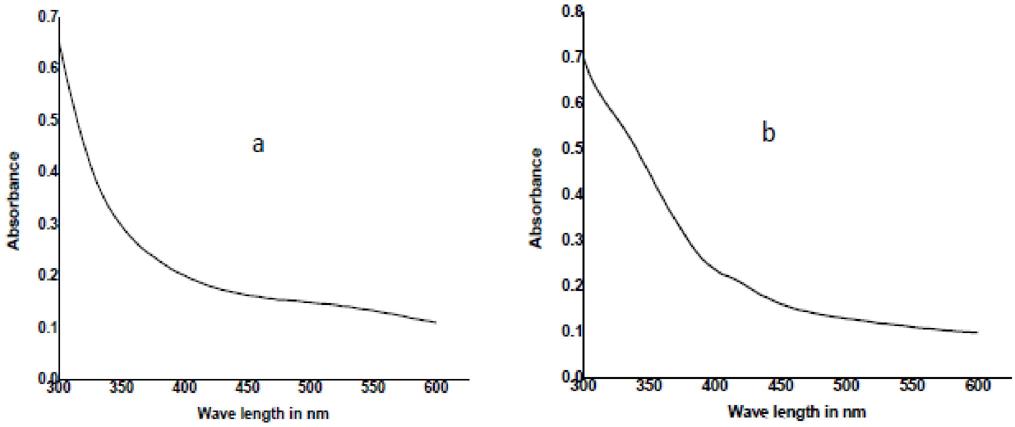

Figure 39: UV-vis spectra of a mixture of dichloromethane-hexane extract and Ag NO_3 (a), dichloromethane-hexane extract (b)

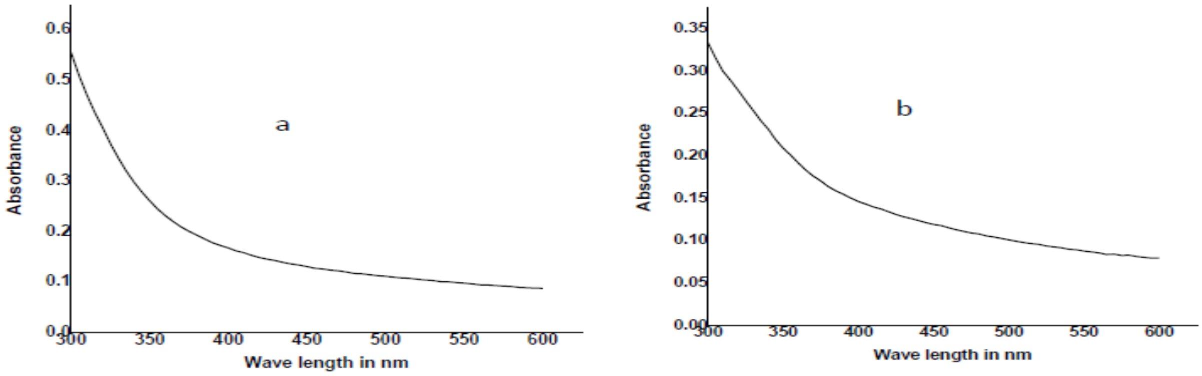

Figure 40: UV-vis spectra of a mixture of hexane extract and Ag NO_3 (a), hexane plant extract (b)

4.4.1 Scanning Electron Microscopy (SEM), Energy Dispersive X-ray (EDX), and Transmission Electron Microscopy (TEM) Analysis

The morphology and elemental composition of synthesized silver nanoparticles were determined using SEM/EDX while the TEM micrograph further revealed the size and general morphology of the nanoparticles. The SEM micrographs for the synthesized nanoparticles are shown in figure 41 for aqueous-Ag NPs, dichloromethane-methanol-Ag NPs (figure 42), dichloromethane-Ag NPs (figure 43), methanol-Ag NPs (figure 44), dichloromethane-ethyl acetate-Ag NPs (figure 45), and ethyl acetate-Ag NPs (figure 46). The SEM images showed that the nanoparticles were generally spherical and others formed aggregates. The particles also had a smooth surface. The elemental analysis of various silver nanoparticles using EDX showed that the percentage of Ag metal in occurrence with other chemical elements was significant in all the synthesized nanoparticles except aqueous-Ag NPs that had 14.01% (figure 47).

In dichloromethane-methanol-Ag NPs the percentage of silver was 81.33%, (figure 48), ethyl acetate-Ag NPs 72.19% (Figure 49), dichloromethane-Ag NPs was 67.26%, (figure 50), methanol-Ag NPs was 56.58% (figure 51), and dichloromethane-ethyl acetate-Ag NPs 69.54% (figure 52). TEM micrographs further revealed the size and shape of the nanoparticles. From the micrographs, the particles were generally spherical. The sizes were determined by measuring the diameter of the images in TEM micrograph using *imageJ* software, and generating histograms, which gave particle size distribution. The TEM micrograph for dichloromethane-methanol-Ag NPs is shown in figure (A), aqueous-Ag NPs figure 54 (A), dichloromethane-Ag NPs figure 55 (A), methanol-Ag NPs figure 56 (A), dichloromethane-ethyl acetate-Ag NPs figure 57 (A), and ethyl acetate-Ag NPs, figure 58 (A).

The histograms generated from measuring the diameters of the nanoparticles in the TEM micrographs revealed that the particles had an average size of 27±12.2 nm for dichloromethane-methanol-Ag NPs figure 53 (B), aqueous-Ag NPs 24.4 ± 8.8 nm figure 54 (B), dichloromethane-Ag NPs 22.8± 17.5 nm figure 55 (B), methanol-Ag NPs 31.8 ± 11.4 nm figure 56 (B), dichloromethane-ethyl acetate-Ag NPs 34.7±20.3 nm figure 57 (B), and ethyl acetate-Ag NPs (75.3± 19.7 nm) figure 58 (B). Appendix 25-30 shows the *imageJ* data used in obtaining various sizes of the biosynthesized Ag NPs.

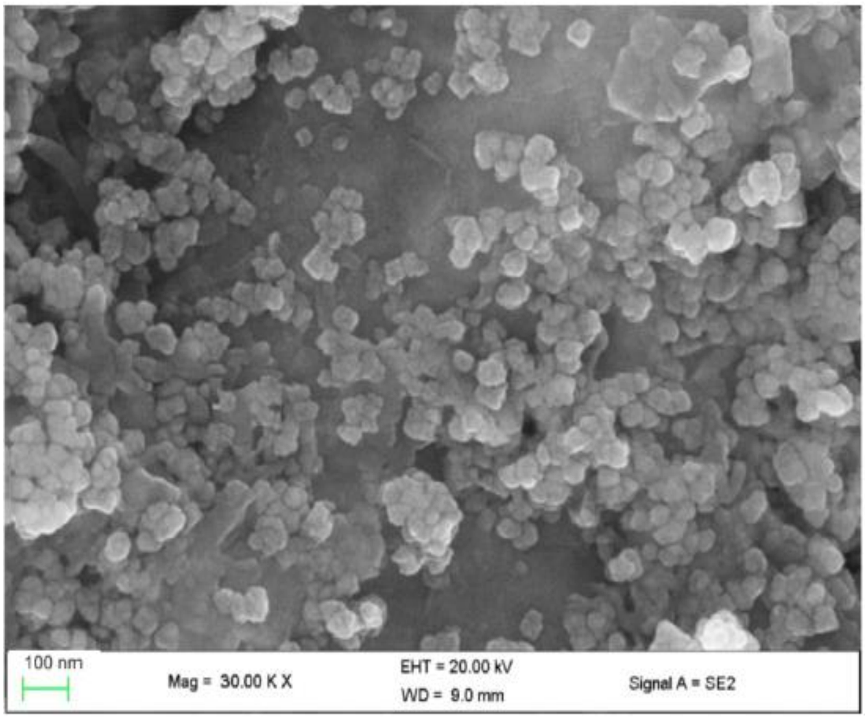

Figure 41: SEM micrograph aqueous-Ag NPs

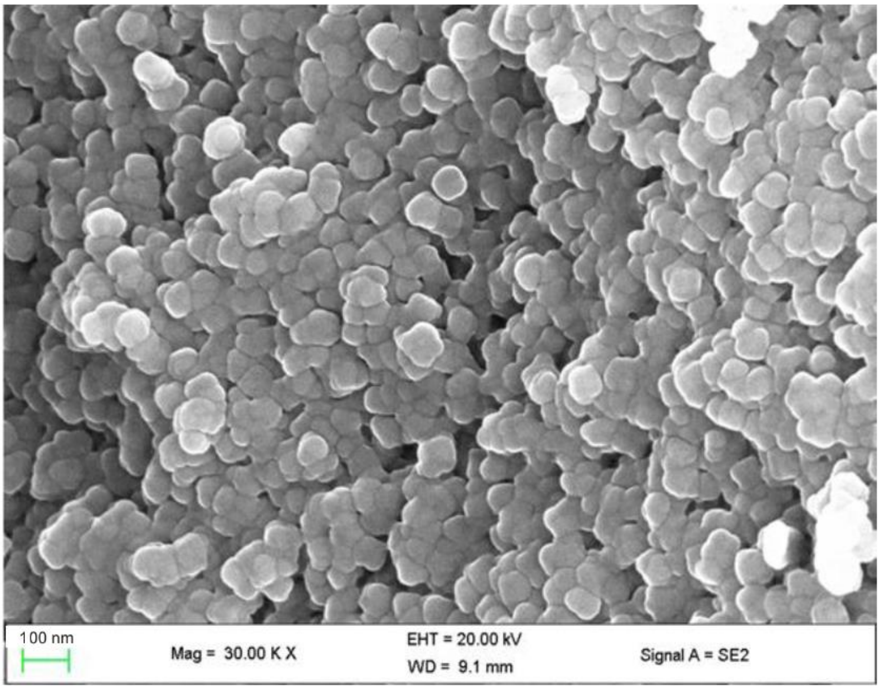

Figure 42: SEM micrograph dichloromethane-methanol-Ag NPs

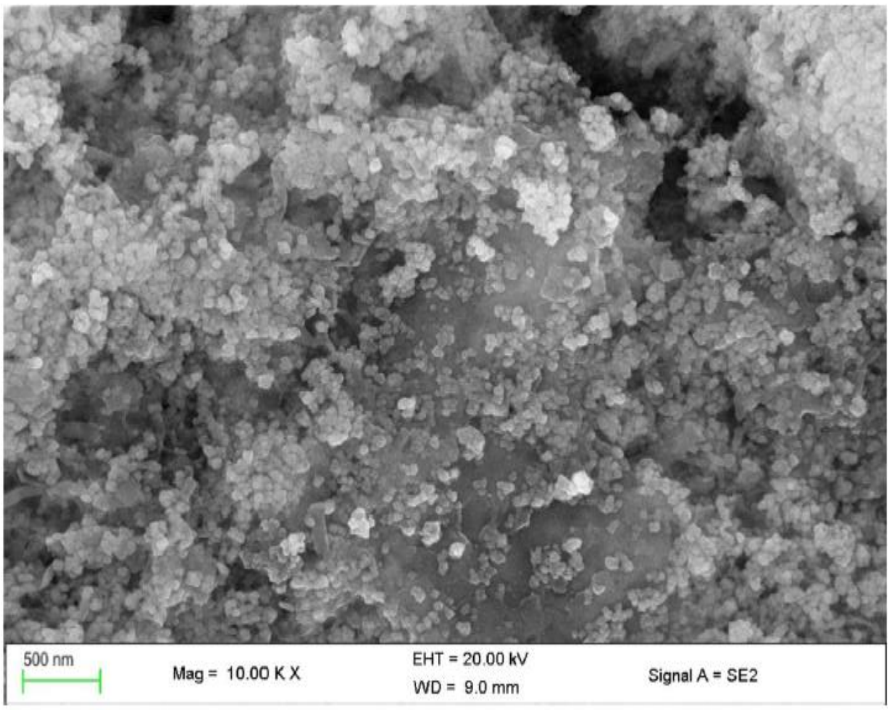

Figure 43: SEM micrograph dichloromethane-Ag NPs

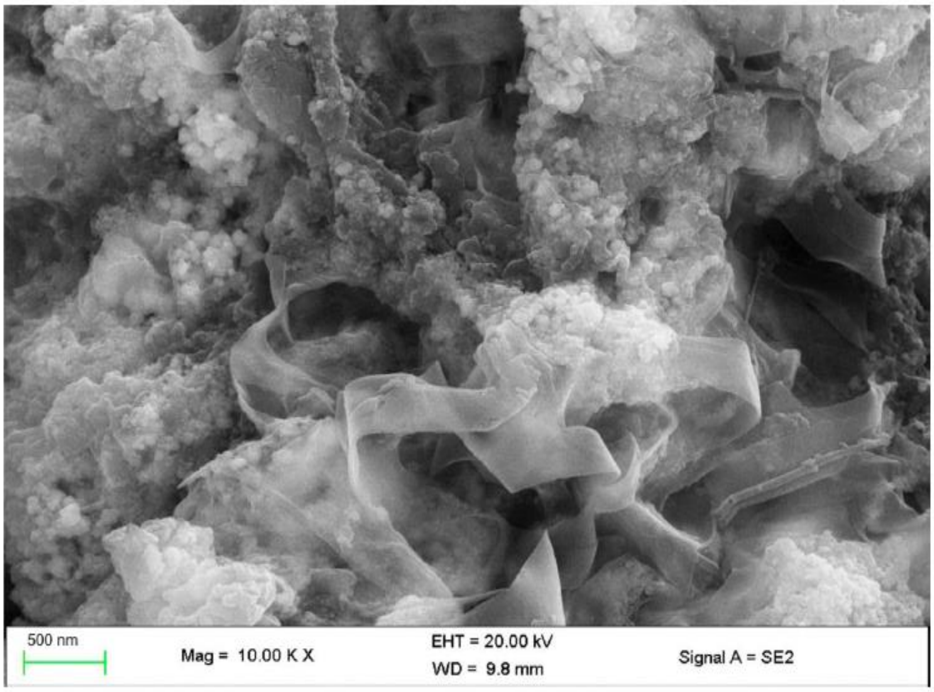

Figure 44: SEM micrograph methanol-Ag NPs

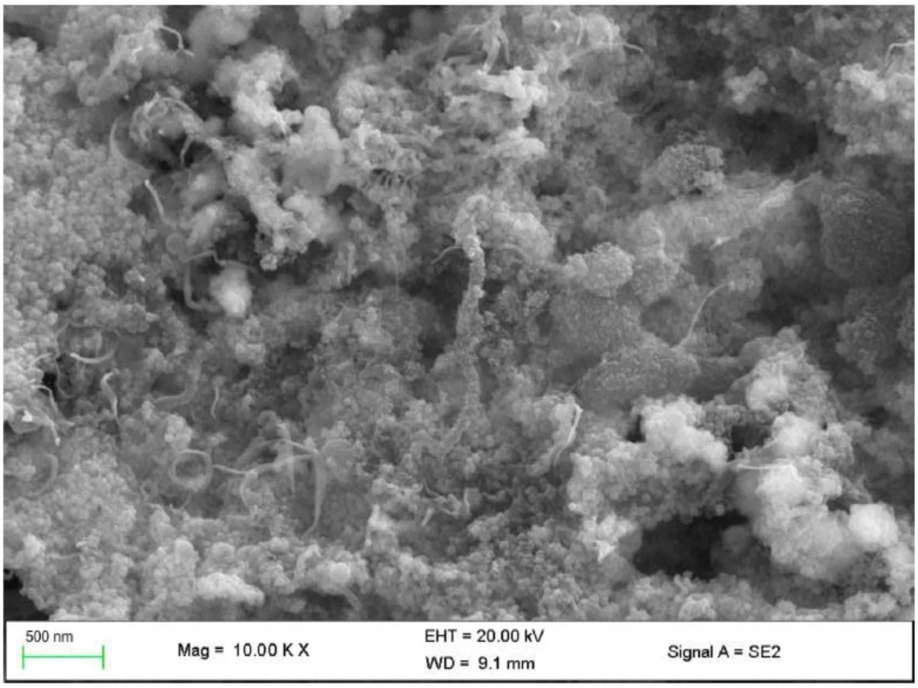

Figure 45: SEM micrograph dichloromethane-ethyl acetate-Ag NPs

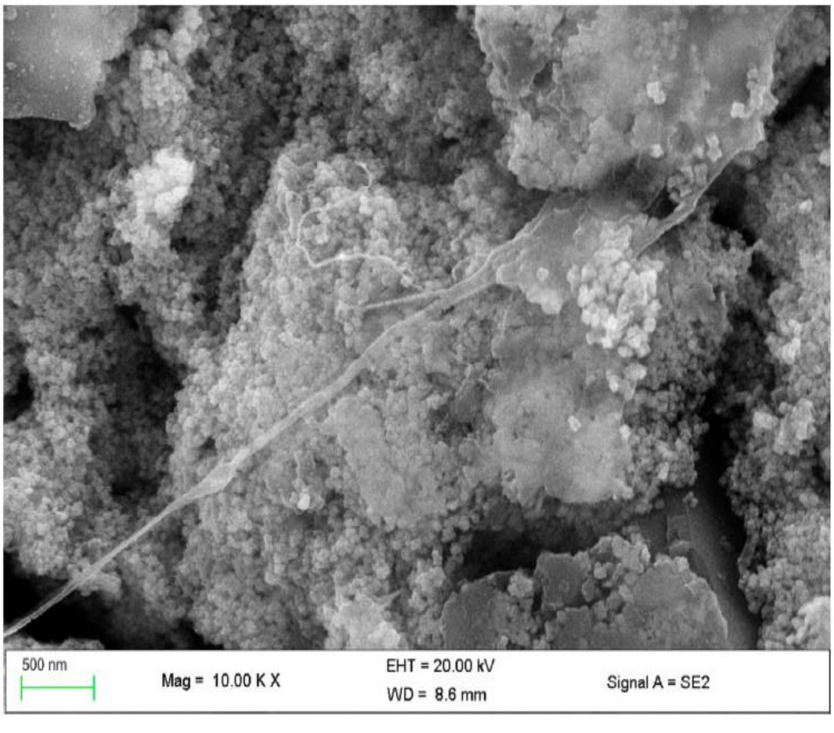

Figure 46: SEM micrograph ethyl acetate-Ag NPs

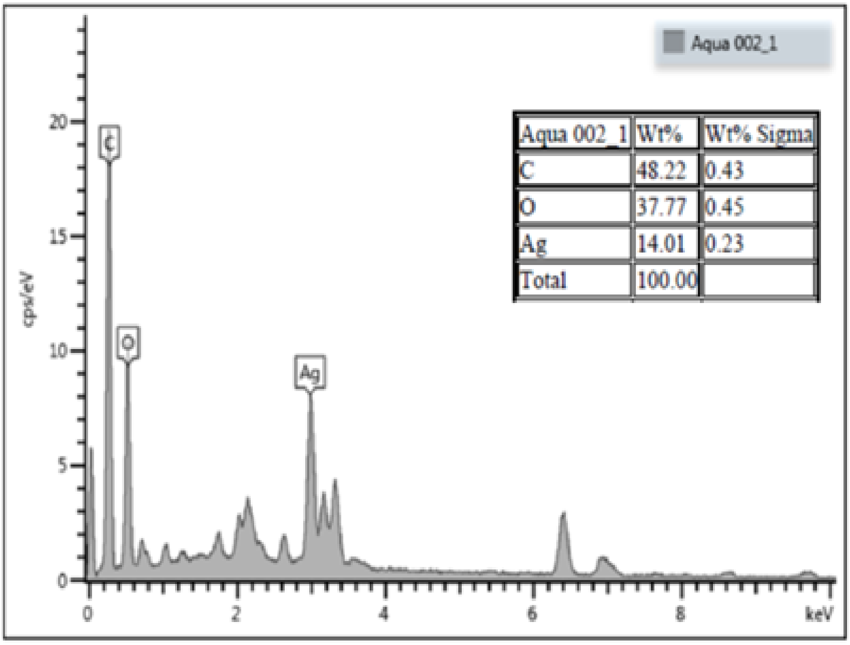

Figure 47: EDX micrograph aqueous-Ag NPs

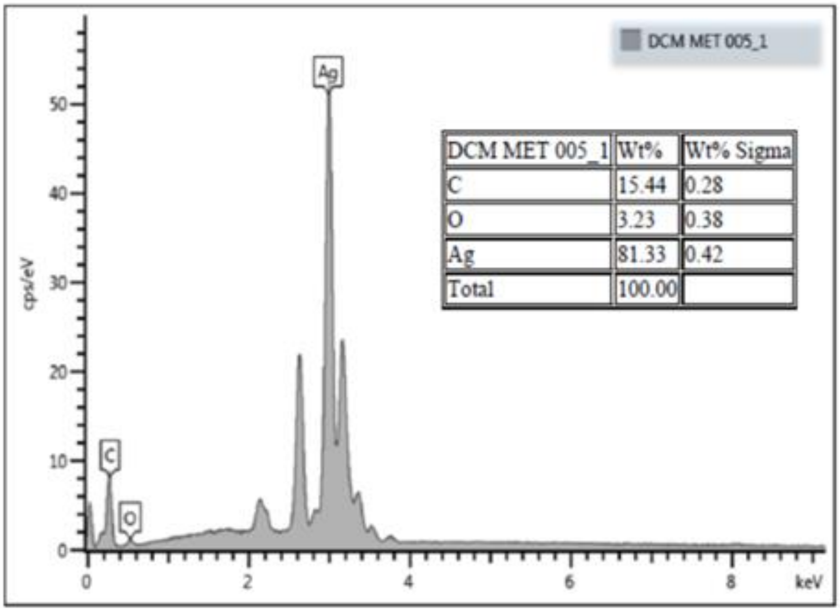

Figure 48: EDX micrograph dichloromethane-methanol-Ag NPs

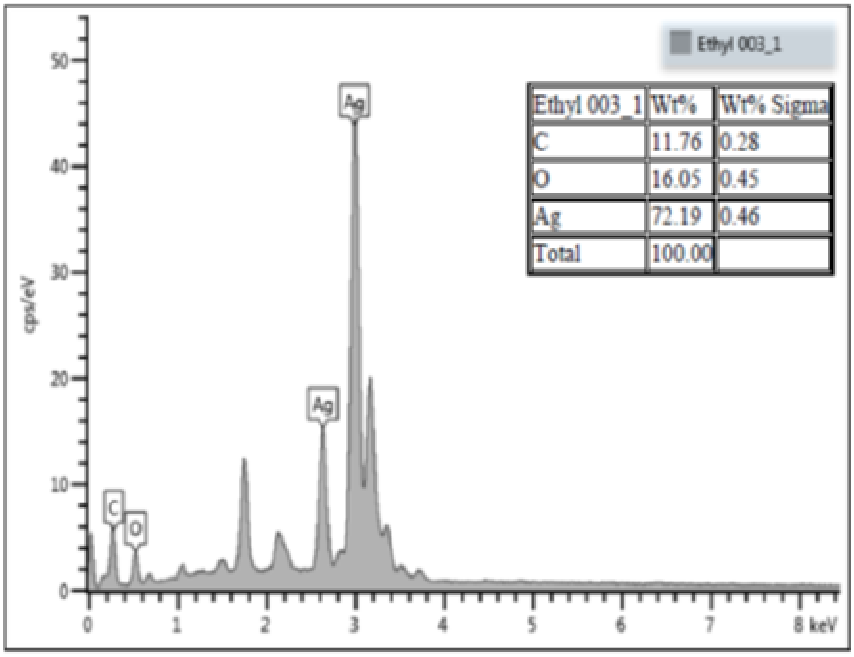

Figure 49: EDX micrograph ethyl acetate-Ag NPs

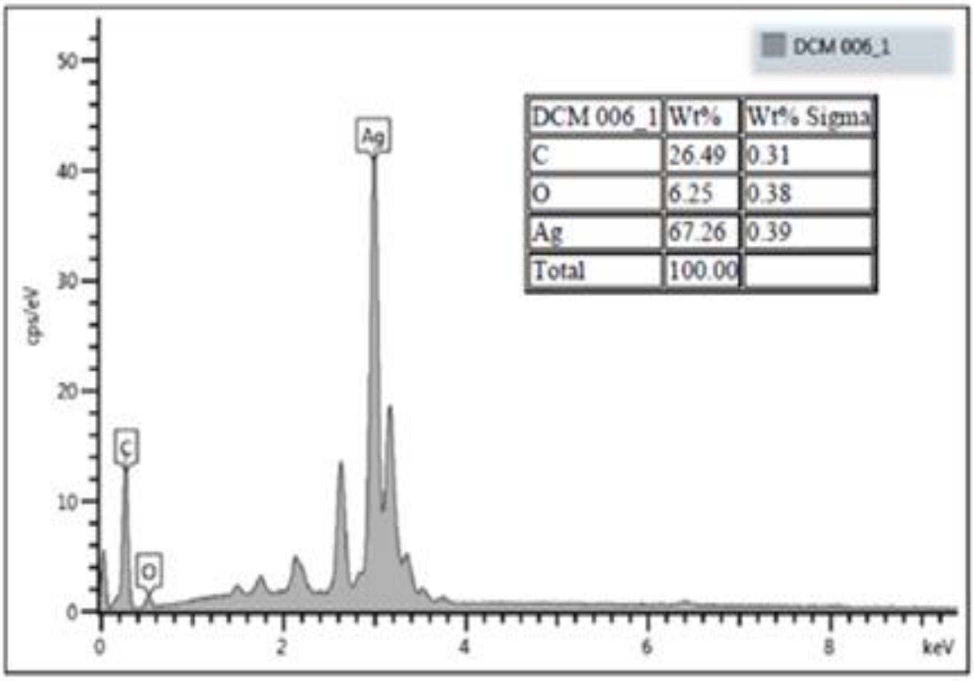

Figure 50: EDX micrograph dichloromethane-Ag NPs

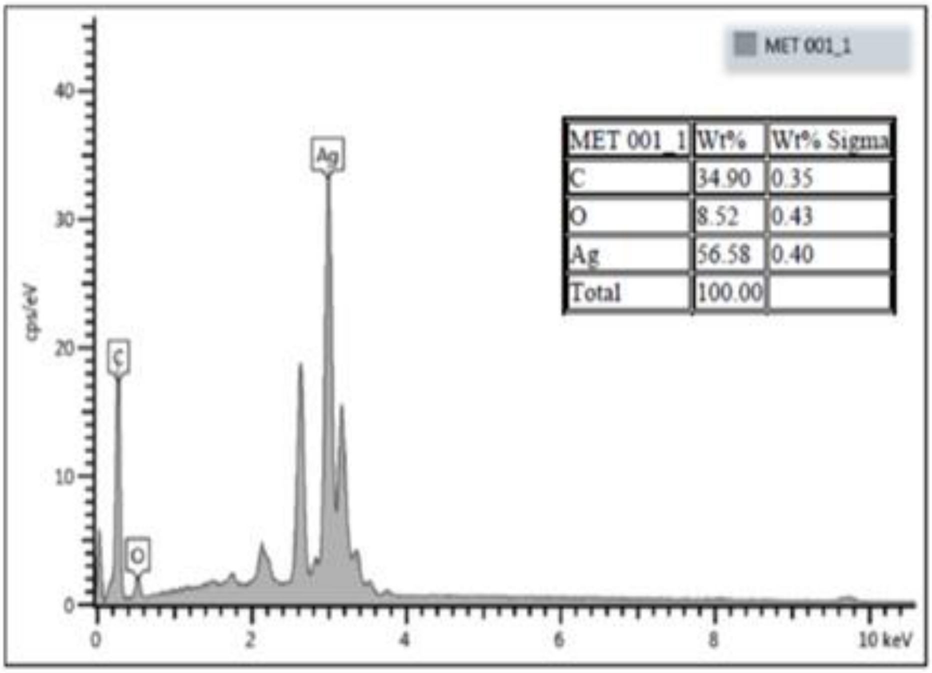

Figure 51: EDX micrograph methanol-Ag NPs

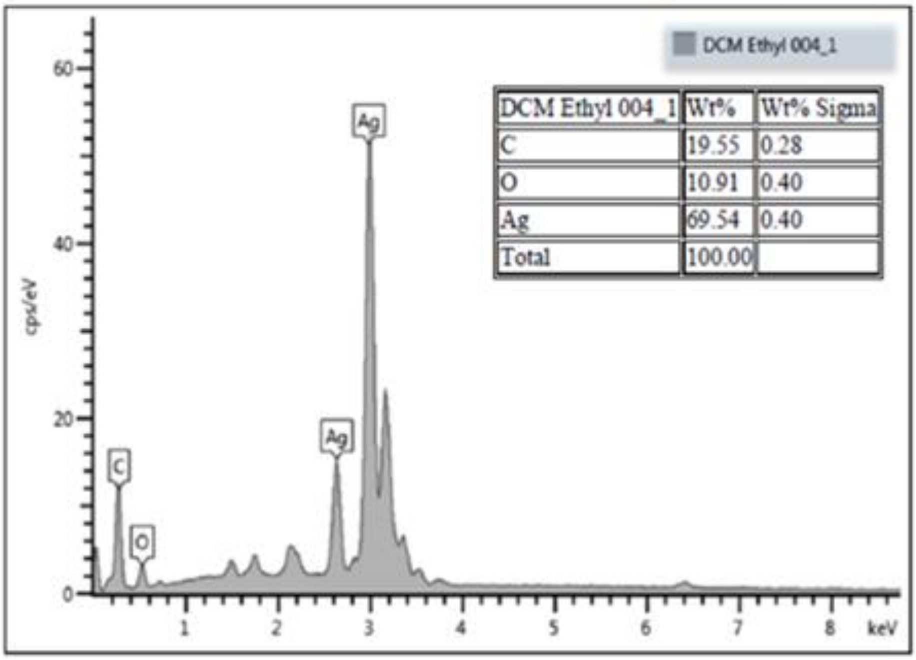

Figure 52: EDX micrograph dichloromethane-ethyl acetate-Ag NPs

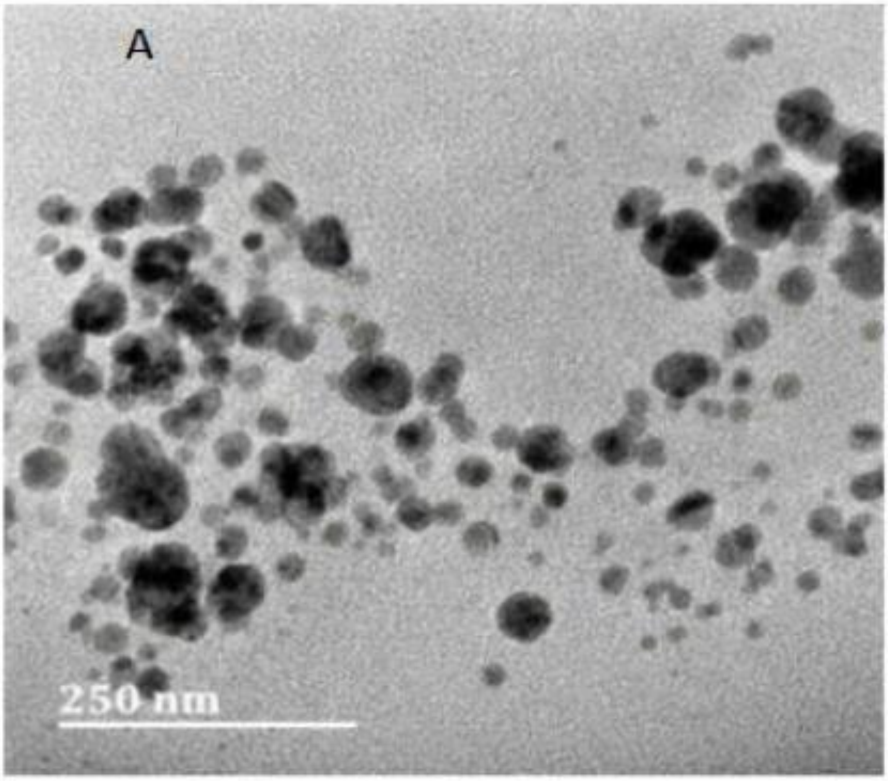

Figure 53 A: TEM Micrograph dichloromethane-methanol-Ag NPs

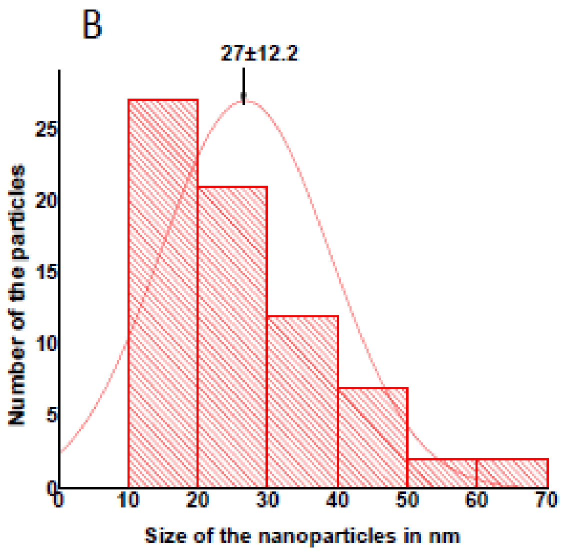

Figure 53 B: Particle size distribution histogram of dichloromethane-methanol-Ag NPs

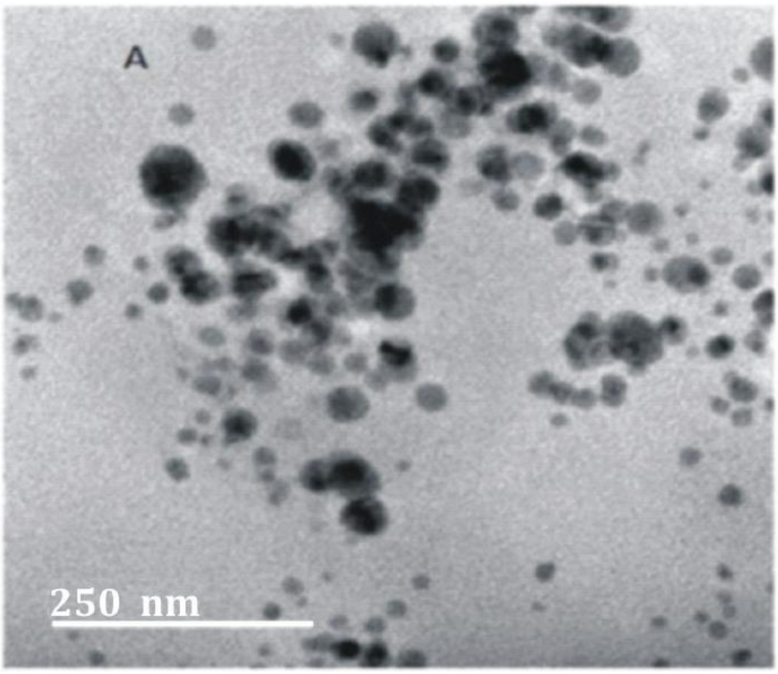

Figure 54 A: TEM Micrograph aqueous-Ag NPs

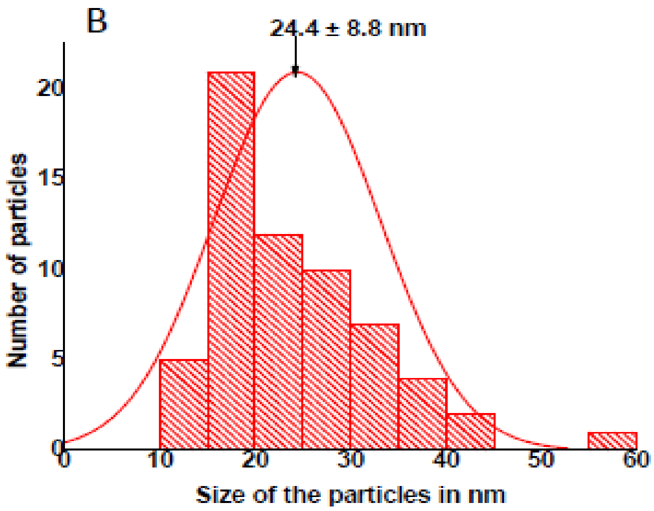

Figure 54 B: Particle size distribution histogram of aqueous- Ag NPs

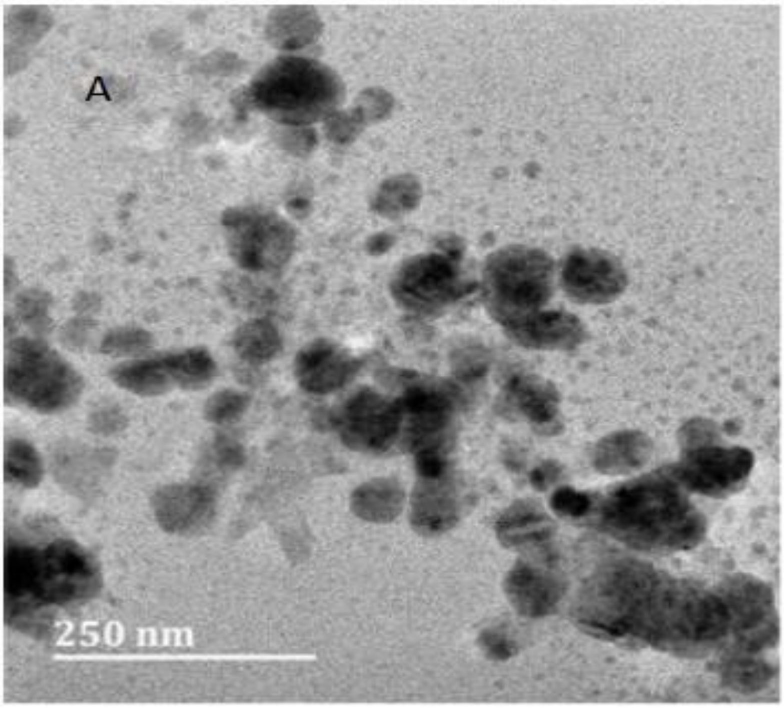

Figure 55 A: TEM micrograph dichloromethane-Ag NPs

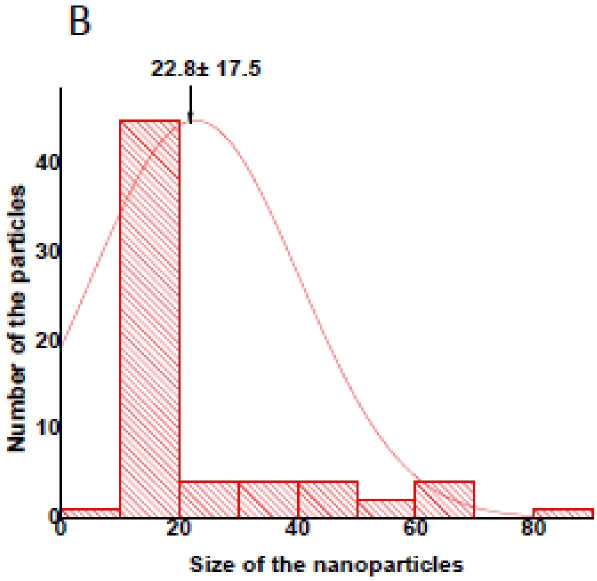

Figure 55 B: Particle size distribution histogram of dichloromethane-Ag NPs

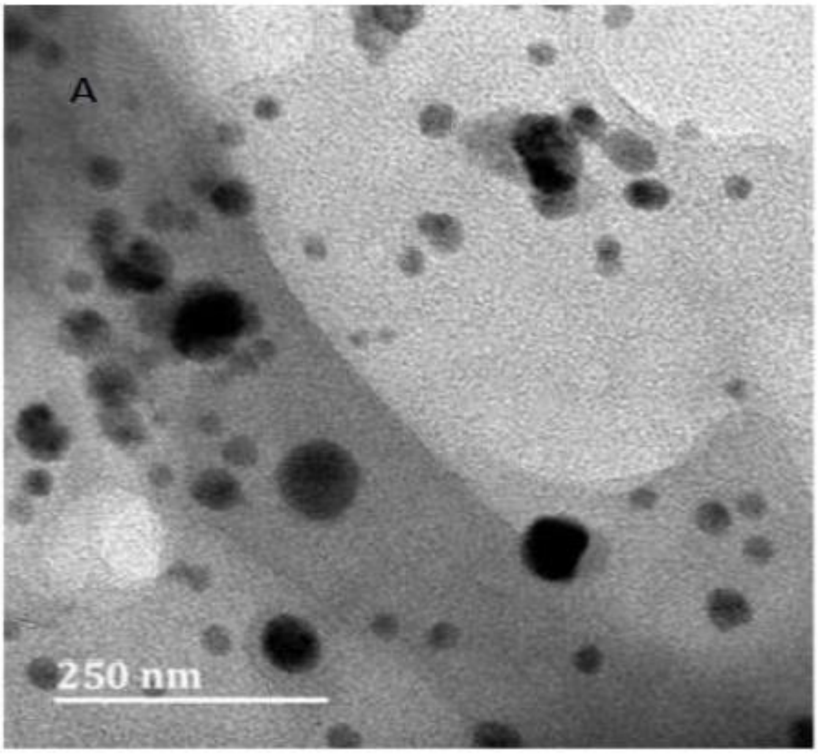

Figure 56 A: TEM micrograph methanol-Ag NPs

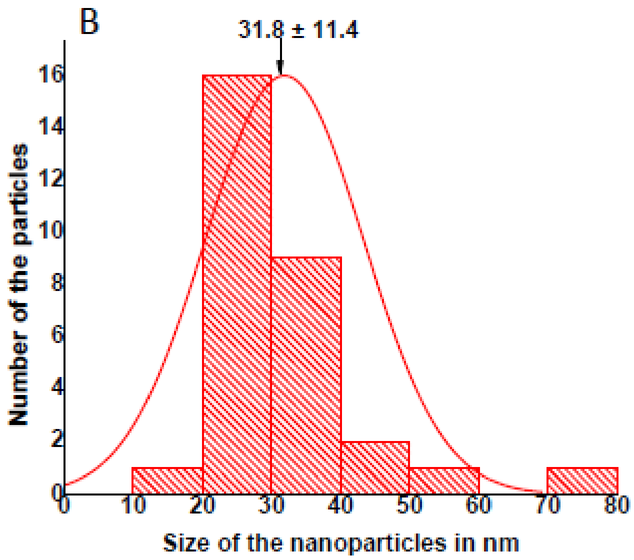

Figure 56 B: Particle size distribution histogram of methanol-Ag NPs

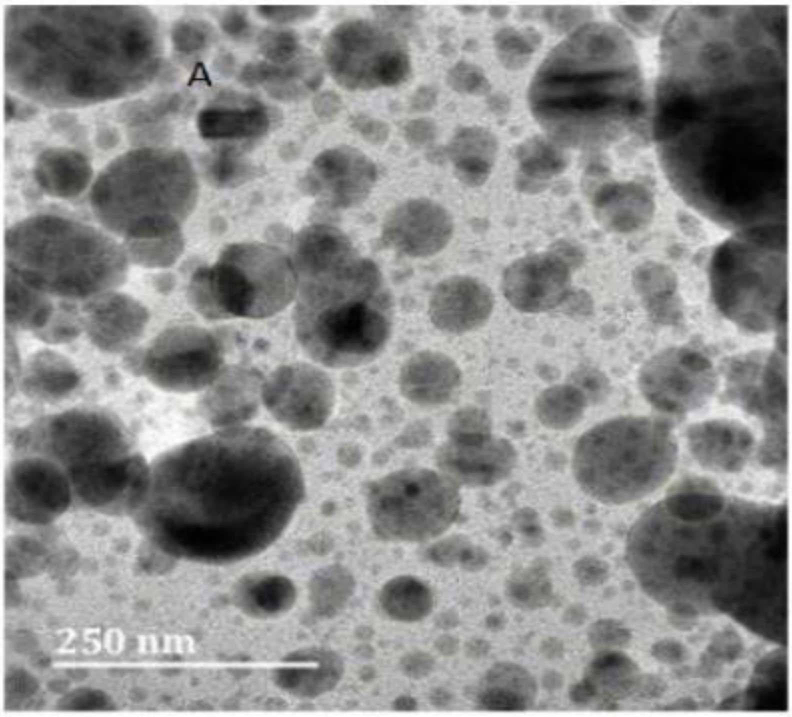

Figure 57 A: TEM micrograph dichloromethane-ethyl acetate-Ag NPs

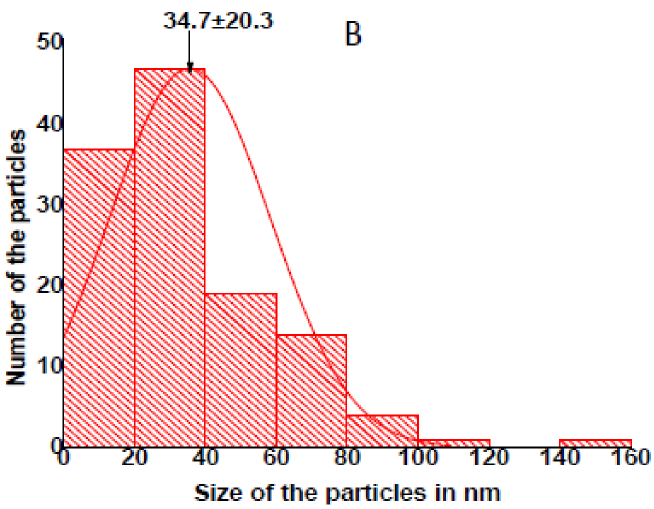

Figure 57 B: Particle size distribution histogram of dichloromethane-ethyl acetate-Ag NPs

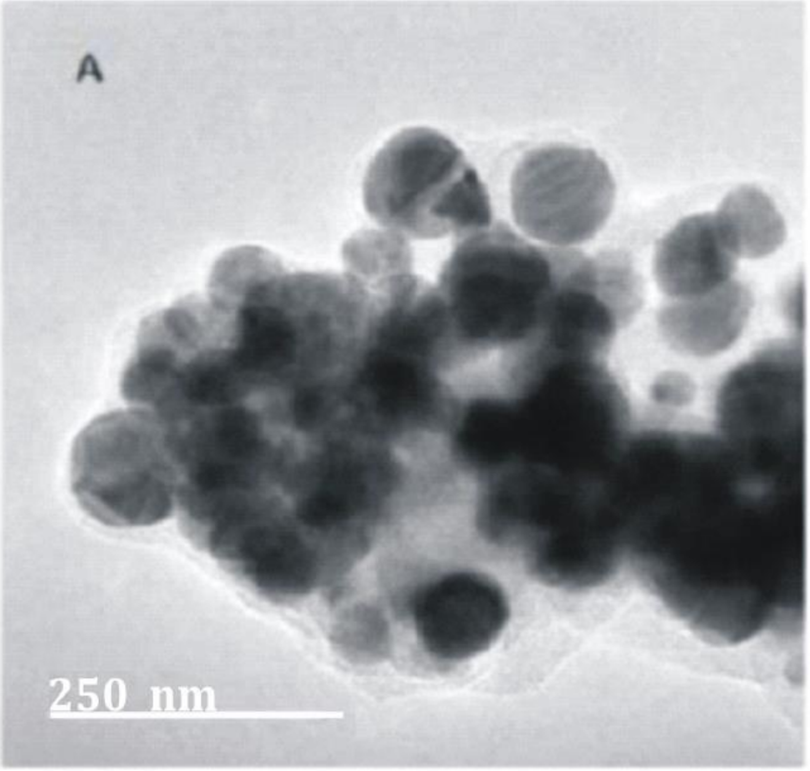

Figure 58 A: TEM micrograph ethyl acetate-Ag NPs

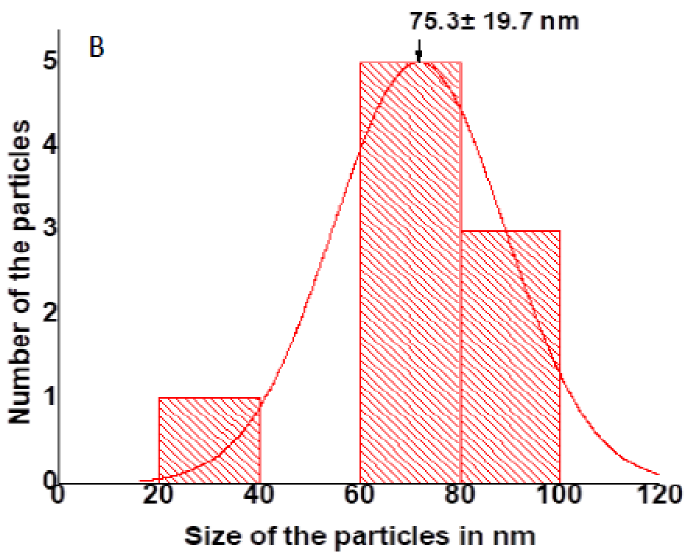

Figure 58 B: Particle size distribution histogram of ethyl acetate-Ag NPs

4.4.2 Fourier-Transform Infrared Spectroscopy (FTIR) analysis

The FTIR spectra figures (59-65) showed the absorption bands for various functional groups on the surface of the synthesized silver nanoparticles. These functional groups belong to various phytochemicals present in *C. cinerariaefolium* extracts and could have been responsible for the synthesis of the nanoparticles. The phytochemicals interacted with silver ions in silver nitrate thus reducing the ions to silver nanoparticles and stabilizing/capping the formed nanoparticles. The absorption bands for all the nanoparticles were similar with slight shifts in the peaks indicating that the same phytochemicals were responsible for reducing and stabilization of the nanoparticles.

The strong stretching vibrations of the hydroxyl group (-OH) belonging to phenolic compounds was exhibited by absorption bands at 3489.59 cm^{-1} in dichloromethane-Ag NPs, 3490.95 cm^{-1} and 3341.90 cm^{-1} in aqueous-Ag NPs, 3490.95 cm^{-1} in dichloromethane-methanol-Ag NPs, 3472.83 cm^{-1} in methanol-Ag NPs, 3472.88 cm^{-1} in dichloromethane-ethyl acetate-Ag NPs, and 3469.54 cm^{-1} in ethyl acetate-Ag NPs (Vanaja *et al.,* 2013). Likewise the weak stretching vibrations of hydroxyl group (-OH) was observed at 3217.80 cm^{-1} in dichloromethane-Ag NPs, 3166.28 cm^{-1} in aqueous-Ag NPs, 3209.29 cm^{-1} in dichloromethane-methanol-Ag NPs, 3214.15 cm^{-1} in ethyl acetate-Ag NPs, and 3190.67 cm^{-1} in both dichloromethane-ethly acetate-Ag NPs and methanol-Ag NPs (Vanaja *et al.,* 2013). The bands at 2384.74 cm^{-1}, 2394.29 cm^{-1}, 2411.86 cm^{-1}, 2381.12 cm^{-1} belonging to dichloromethane-Ag NPs, methanol-Ag NPs, dichloromethane-ethyl acetate–Ag NPs, and ethyl acetate-Ag NPs respectively were attributed to the presence of C=O band (Dorranian *et al.,* 2012).

The bands at 1633.05 cm^{-1}, 1629.52 cm^{-1}, 1617.64 cm^{-1}, 1646.61 cm^{-1}, 1633.05 cm^{-1}, and 1626.20 present in dichloromethane-Ag NPs, aqueous-Ag NPs, dichloromethane-methanol- Ag NPs, methanol-Ag NPs, dichloromethane-ethyl acetate- Ag NPs, and ethyl acetate-Ag NPs respectively are ascribed to C=C present in aromatic compounds of the phytochemicals (Raghunandan *et al.,* 2010). Moreover, C-O stretching vibration for carbonate group was observed at the bands 1405.08 cm^{-1}, 1409.93 cm^{-1}, 1412.69 cm^{-1}, 1404.05,cm^{-1}, 1405.08 cm^{-1}, 1404.48 cm^{-1} present in dichloromethane-Ag NPs, aqueous-Ag NPs, dichloromethane-methanol-Ag NPS, dichloromethane-ethyl acetate-Ag NPs, methanol-Ag NPs, and ethyl acetate-Ag NPs respectively (Meejoo *et al.,* 2006). Presence of carboxylic acid group was ascertained by the bands at 1109.32 cm^{-1}, 1104.90 cm^{-1}, 1104.75 cm^{-1}, 1109.32 cm^{-1}, 1122.88 cm^{-1}, and 1104.90 cm^{-1} present in dichloromethane-Ag NPs, aqueous-Ag NPs, dichloromethane-methanol- Ag NPs, methanol-Ag NPs, dichloromethane-ethyl acetate- Ag NPs, and ethyl acetate-Ag NPs respectively (Pirtarighat *et al.,* 2019).

The peaks at 505.93 cm^{-1}, 505.04 cm^{-1}, 500.88 cm^{-1}, 518.64 cm^{-1}, 505.93 cm^{-1}, and 504.81 cm^{-1} present in dichloromethane-Ag NPs, aqueous-Ag NPs, dichloromethane-methanol- Ag NPs, methanol-Ag NPs, dichloromethane-ethyl acetate- Ag NPs, and ethyl acetate-Ag NPs respectively, represent stretching vibrations of the metal-oxygen bond (Ag-

O) (Tripathi *et al.*, 2011). The band 616.86 cm^{-1} in dichloromethane-methanol- Ag NPs also belong to stretching vibrations of the metal–oxygen bond (Ag-O) (Tripathi *et al.*, 2011). Appendix 32 shows the wave numbers and percentage transmittance of various Ag NPs.

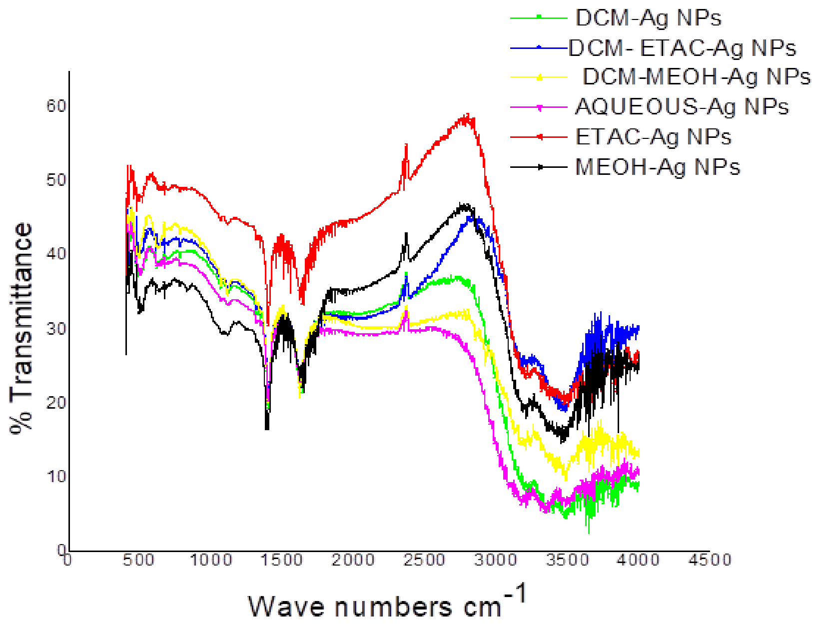

Figure 59: FTIR spectra for all synthesized Ag NPs

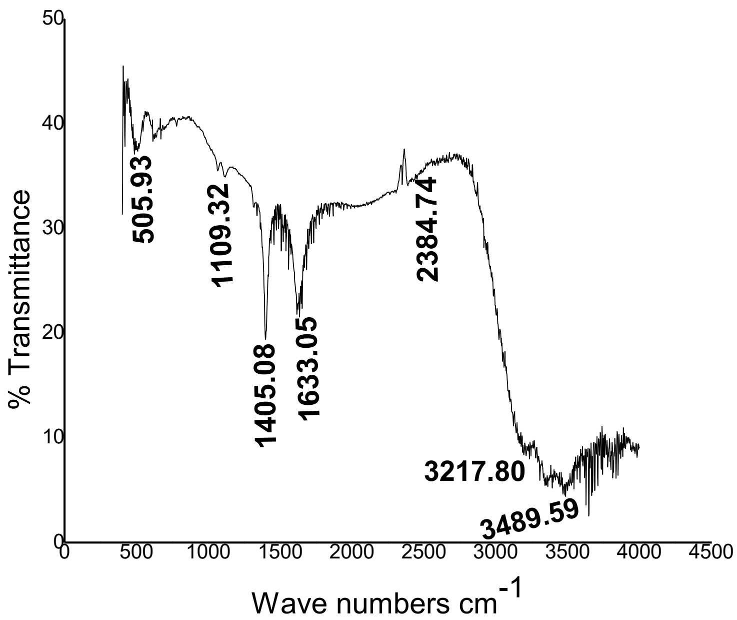

Figure 60: FTIR spectra for dichloromethane-Ag NPs

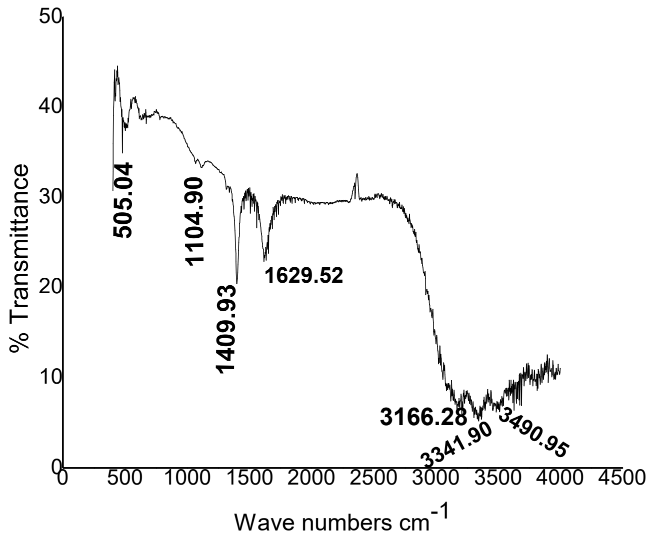

Figure 61: FTIR spectra for aqueous-Ag NPs

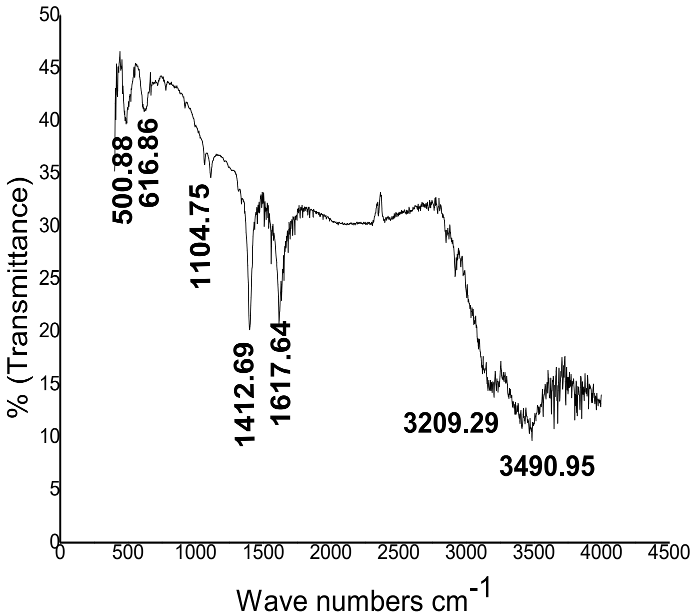

Figure 62: FTIR spectra for dichloromethane-methanol-Ag NPs

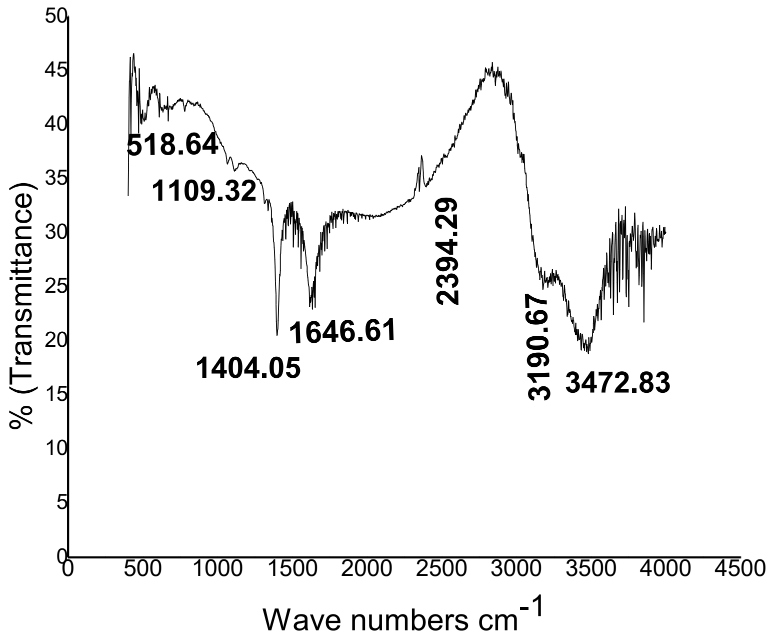

Figure 63: FTIR spectra for methanol-Ag NPs

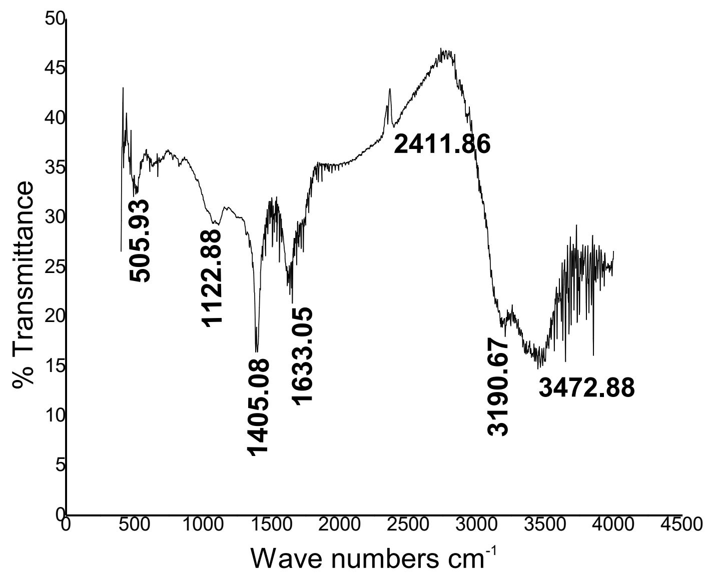

Figure 64: FTIR spectra for dichloromethane-ethyl acetate-Ag NPs

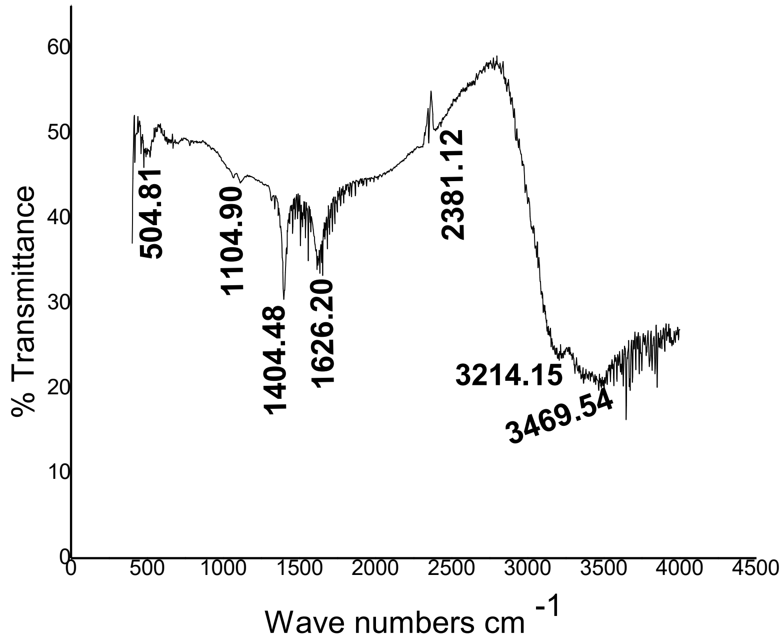

Figure 65: FTIR spectra for ethyl acetate-Ag NPs

4.5 Phytochemical analysis of crude extracts used in synthesis silver nanoparticles

Phytochemical results of crude extracts used in the synthesis of Ag NPs are presented in Table 7. From the results, dichloromethane, aqueous, and dichloromethane-ethyl acetate extracts had the majority of the phytochemicals i.e saponins, flavonoids, alkaloids, phenols, tannins, and glycosides. Methanol extract had flavonoids, alkaloids, phenols, tannins, and glycosides while dichloromethane-methanol extract had flavonoids, phenols, tannins, and glycosides. On the other hand, ethyl acetate extract had flavonoids, phenols, and tannins. Hexane and dichloromethane-hexane extracts had the least phytochemicals.

Table 7: Phytochemical analysis of crude extracts used in silver nanoparticle synthesis

Phytochemicals tested	Dichloromethane extract	Dichloromethane-methanol extract	Dichloromethane ethyl acetate extract	Ethyl acetate extract	Aqueous extract	Methanol extract	Dichloromethane-Hexane extract	Hexane extract
Saponins	+	-	+	-	+	-	-	-
Flavanoids	+	+	+	+	+	+	+	+
Alkaloids	+	-	+	-	+	+	-	-
Phenols	+	+	+	+	+	+	-	-
Tannins	+	+	+	+	+	+	-	-
Glycosides	+	+	+	-	+	+	+	+
Terpenoids	-	-	-	-	-	-	-	-

[+] Present, [-]Absent

4.6 Bioassay of biosynthesized Ag NPs

4.6.1 Disc diffusion assay

Results of disc diffusion assay of the Ag NPs against selected bacteria are presented in Table 8. The values are the mean of three experiments ± S.D. Within a column, the inhibition zones of extracts sharing the same letters were not significantly different while those with different letters are significantly different (*α=0.05*, One way ANOVA). From the results, the nanoparticles were susceptible to all the bacteria. Silver nanoparticles were also more active than silver ions and the plant extracts used in the synthesis of the nanoparticles. Appendix 31 shows the bacterial bioassay results.

Table 8: Bioassay results of synthesized Ag NPs, plant extracts (control) and silver nitrate (control)

	Zone of inhibition in mm at 500 µg/ml			
Extracts	***MRSA***	***S. aureus***	***P.aeruginosa***	***S. sonnie***
Dichloromethane-Ag NPs	7.3 ± 0.2^{b}	9.7 ± 0.8^{c}	13.3 ± 0.6^{a}	9.5 ± 0.5^{c}
Dichloromethane plant extract	6 ± 0^{h}	6 ± 0^{h}	6 ± 0^{h}	6 ± 0^{h}
Aqueous-Ag NPs	7.2 ± 0.3^{a}	9.8 ± 0.3^{b}	9 ± 1^{bd}	8.3 ± 0.5^{d}
Aqueous plant extract	6 ± 0^{h}	6 ± 0^{h}	6 ± 0^{h}	6 ± 0^{h}
Dichloromethane-methanol-Ag NPs	7.5 ± 0.3^{a}	10.8 ± 0.8^{c}	9.3 ± 0.6^{c}	9.5 ± 0.5^{c}
Dichloromethane-methanol extract	6 ± 0^{h}	6 ± 0^{h}	6 ± 0^{h}	6 ± 0^{h}
Methanol-Ag NPs	7.2 ± 0.3^{a}	8.3 ± 0.3^{b}	9.5 ± 0.5^{c}	8.2 ± 0.3^{b}
Methanol extract	6 ± 0^{h}	6 ± 0^{h}	6 ± 0^{h}	6 ± 0^{h}
Dichloromethane-Ethyl acetate-Ag NPs	6.7 ± 0.2^{a}	8.2 ± 0.3^{b}	7.8 ± 0.3^{b}	8 ± 0.5^{b}
Dichloromethane-ethly acetate extract	6 ± 0^{h}	6 ± 0^{h}	6 ± 0^{h}	6 ± 0^{h}
Ethyl acetate-Ag NPs	6.6 ± 0.2^{a}	7.2 ± 0.2^{b}	7.3 ± 0.3^{b}	7.4 ± 0.4^{b}
Ethyl-acetate extract	6 ± 0^{h}	6 ± 0^{h}	6 ± 0^{h}	6 ± 0^{h}
Aqueous silver nitrate	6.4 ± 0.1^{a}	7.1 ± 0.1^{b}	7 ± 0.5^{ab}	7.1 ± 0.4^{b}
Dmso+distilled H_2O [Q]	6 ± 0^{h}	6 ± 0^{h}	6 ± 0^{h}	6 ± 0^{h}
Chloramphenicol [P]	28 ± 1^{f}	22.7 ± 1.2^{d}	22 ± 2^{d}	30.5 ± 1.3^{g}

*Within a column similar letters show no significant differences while different letters show a significant difference. [P] Positive control, [Q] Negative control

The images of the bioassay of the synthesized Ag NPs against *MRSA* are shown in figure 66, against *S. aureus* (figure 67), against *P.aeruginosa* (figure 68) and against *S.*

sonnie (figure 69). A summary of the contents in the bioassay images are presented in Table 9.

Table 9: Key to contents in the bioassay images of synthesized-Ag NPs against selected bacteria

Content in the images	Actual names
AgNP1	Ethyl acetate -Ag NPs
AgNP2	Methanol-Ag NPs
AgNP3	Dichloromethane-Ag NPs
AgNP4	Dichloromethane-Methanol-Ag NPs
AgNP5	Dichloromethane Ethyl acetate-Ag NPs
AgNP6	Aqueous-Ag NPs

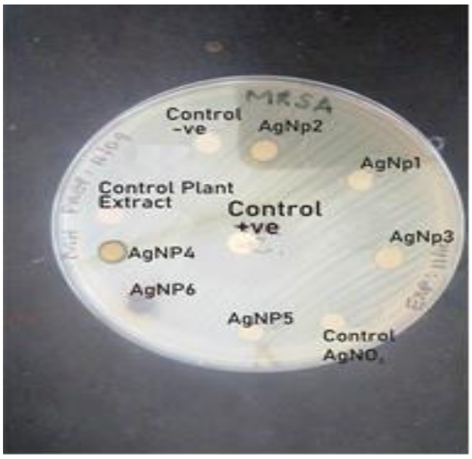

Figure 66: Screening of the Ag NPs at concentration of 500 µg/ml against *MRSA*

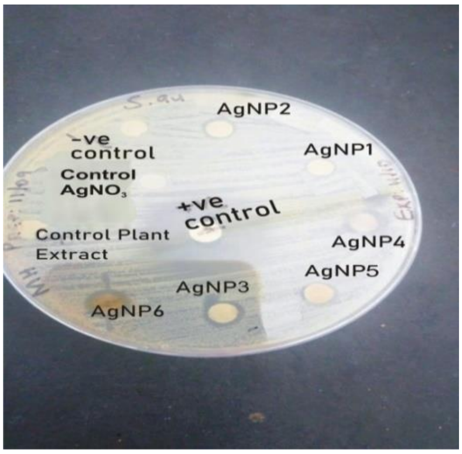

Figure 67: Screening of the Ag NPs at concentration of 500 µg/ml against *S. aureus*

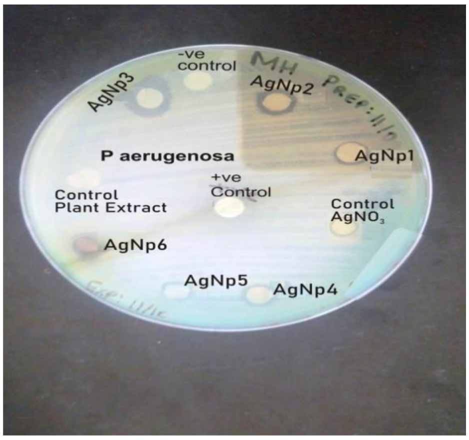

Figure 68: Screening of the Ag NPs at concentration of 500 µg/ml against *P. aeruginosa*

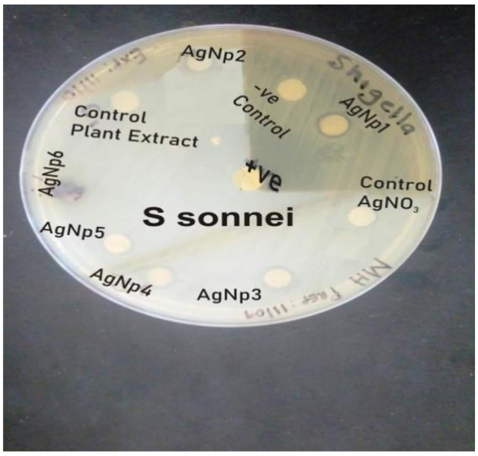

Figure 69: Screening of the Ag NPs at concentration of 500 µg/ml against *S. sonnei*

4.6.2 Minimum inhibitory concentration (MIC)

Silver nanoparticles that caused an inhibition zone of above 10 mm mean ± S.D were subjected to MIC. These were dichloromethane-Ag NPs against *P. aeruginosa* and dichloromethane-methanol-Ag NPs against *S.aureus.* The serial dilutions of the nanoparticles was done in a 96 well plate as presented in figure 70 for dichloromethane-Ag NPs and figure 71 for dichloromethane-methanol-Ag NPs. From the observation made in the 96 well plates, the MIC for dichloromethane-Ag NPs against *P. aeruginosa* was 15.625 µg/ml while dichloromethane-methanol-Ag NPs against *S. aureus* was 31.25 µg/ml. For the positive control, the MIC was 6.25 µg/ml against *P. aeruginosa* and 3.125 µg/ml against *S. aureus.*

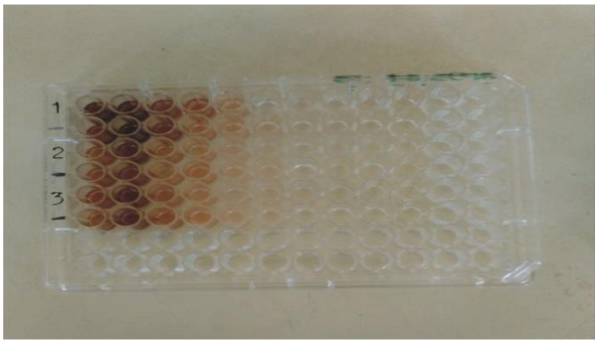

Figure 70: MIC determination of dichloromethane-Ag NPs against *P. aeruginosa*

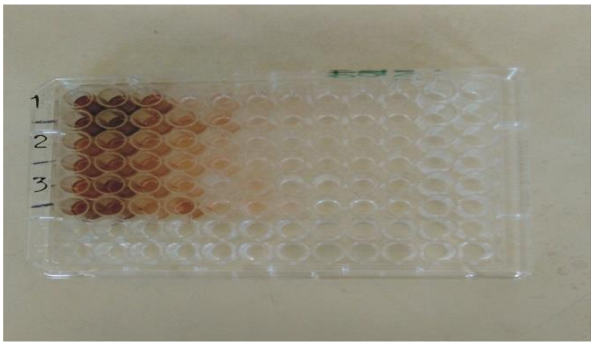

Figure 71: MIC determination of dichloromethane-methanol-Ag NPs against *S. aureus*

4.7 Cytotoxicity assay

Cytotoxicity assay was performed on pure compounds as a mixture (1:1:1) since they had shown more activity than individually isolated compounds on the selected bacteria. From the results, the compounds had an IC_{50} of 74.45 µg/ml compared to the positive control doxorubicin which had an IC_{50} of 11.82 µg/ml (figure 72). The assay was also performed on synthesized nanoparticles, extracts used in the synthesis of the nanoparticles and aqueous silver nitrate. Except for dichloromethane extract which had an IC_{50} of 94.01 µg/ml (figure 73), the rest of the plant extracts used in the synthesis were not cytotoxic at 100 µg/ml hence a higher concentration was needed for the IC_{50} to be achieved. This can be observed in figures (73-78).

Compared to the plant extracts used in the synthesis of Ag NPs, the synthesized Ag NPs were more toxic than the plant extracts with dichloromethane-Ag NPs having the lowest IC_{50} of 33.33 µg/ml (figure 73). Aqueous-Ag NPs had IC_{50} of 47.65 µg/ml for (figure 74), dichloromethane-methanol Ag NPs, 50.49 µg/ml (figure 75), methanol-Ag NPs, 65.74 µg/ml (figure 76), dichloromethane-ethyl acetate-Ag NPs, 68.27 µg/ml (figure 77), and ethyl acetate-Ag NPs, 79.60 µg/ml (figure 78). Silver nitrate was more toxic than the synthesized Ag NPs with an IC_{50} of 21.34 µg/ml (figure 79), while the positive control doxorubicin was the most toxic with an IC_{50} of 11.82 µg/ml. Basic calculations for the absorbance values at 540 nm and 720 nm for various extracts and the nanoparticles are shown in (appendix 33-40).

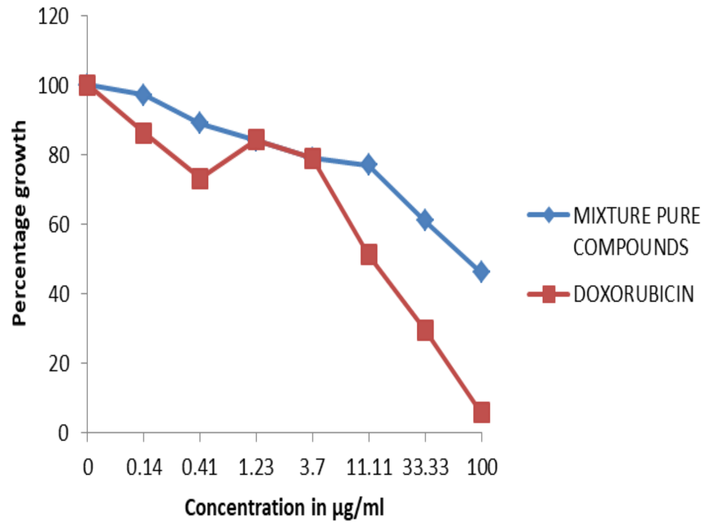

Figure 72: Percentage growth of Vero cells subjected to pure compounds as a mixture (1:1:1) and doxorubicin

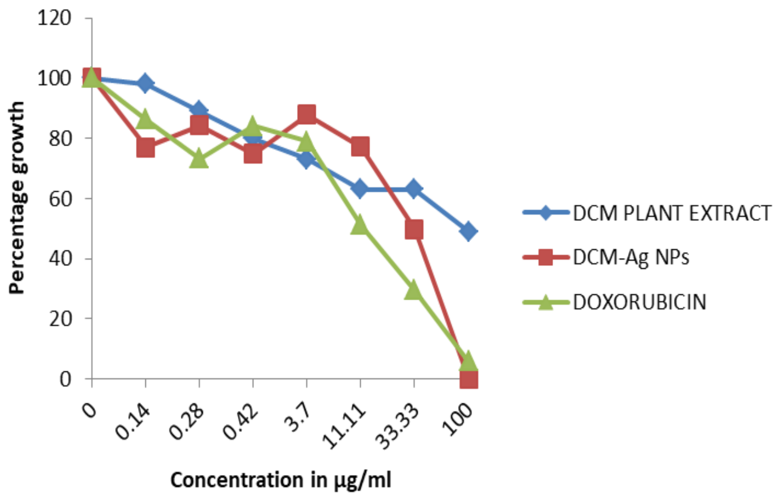

Figure 73: Percentage growth of Vero cells subjected to dichloromethane extract, dichloromethane-Ag NPs and doxorubicin

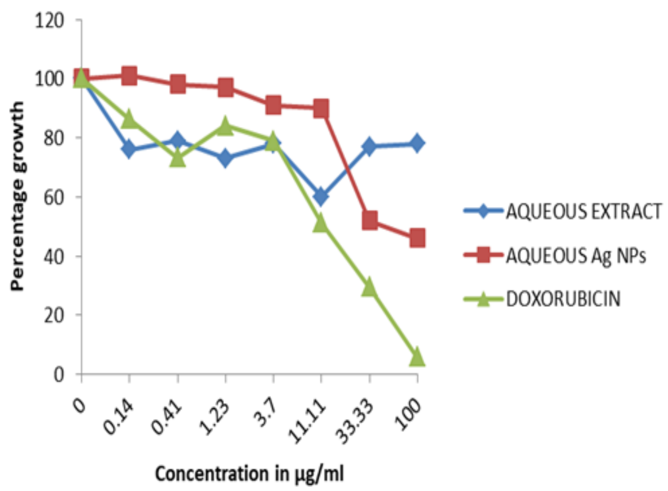

Figure 74: Percentage growth of Vero cells subjected to aqueous extract, aqueous -Ag NPs and doxorubicin

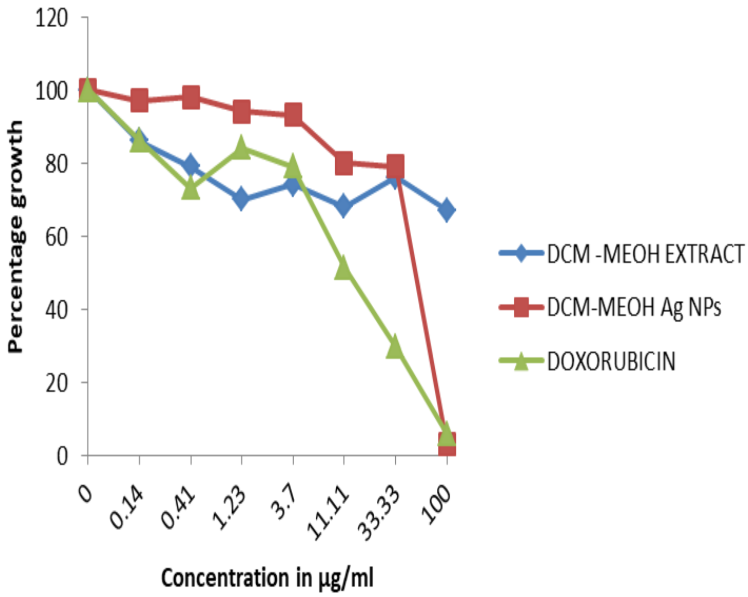

Figure 75: Percentage growth of Vero cells subjected to dichloromethane-methanol extract and dichloromethane-methanol-Ag NPs and doxorubicin

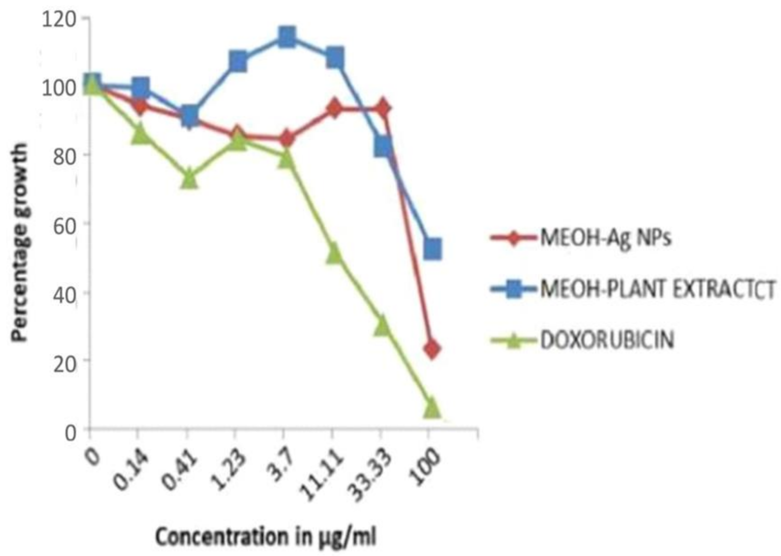

Figure 76: Percentage growth of Vero cells by methanol extract, methanol-Ag NPs and doxorubicin

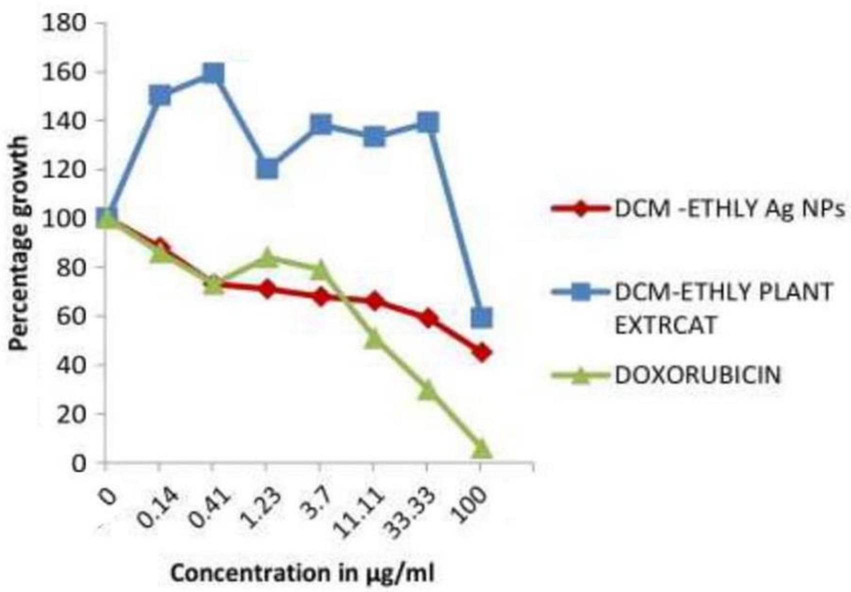

Figure 77: Percetage growth of Vero cells subjected to dichloromethane-ethly acetate extract, dichloromethane-ethly acetate-Ag NPs and doxorubicin

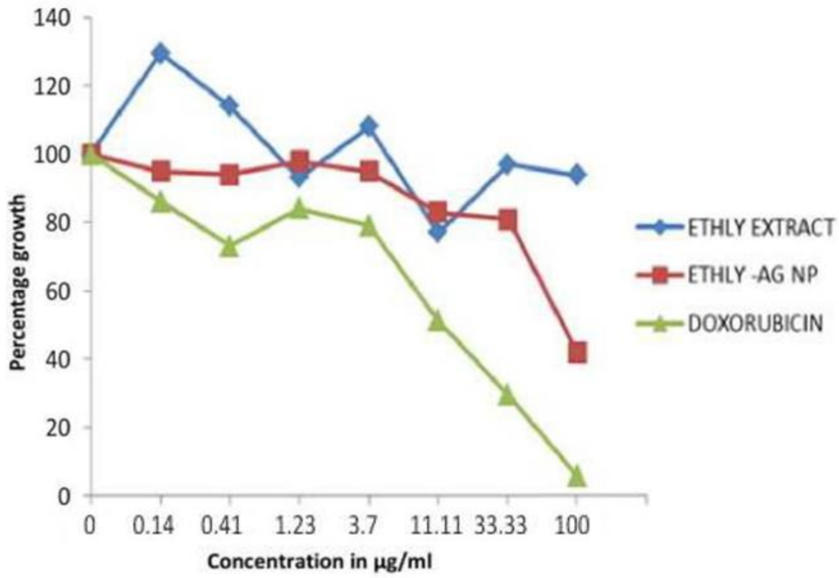

Figure 78: Percentage growth of Vero cells subjected to ethly acetate extract, ethly acetate-Ag NPs and doxorubicin

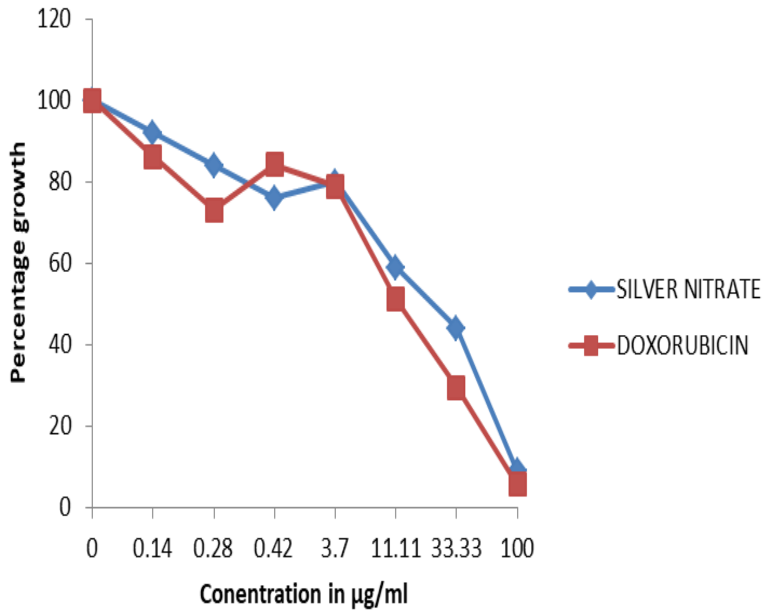

Figure 79: Percentage growth of Vero cells subjected to aqueous silver nitrate and doxorubicin

CHAPTER FIVE

DISCUSSION

5.1 Antibacterial activity of pyrethrum dichloromethane VLC fractions, column fractions, and isolated compounds

Largely, the antimicrobial properties of plant extracts depend on several factors that include environment in which the plant grew, solvent used for the extraction, choice of extraction method, test concentration used, and the method of determination of antimicrobial properties (Valgas *et al.*, 2007). In the current study dichloromethane-methanol crude extract was sequentially extracted with different organic solvents with increasing polarity. The sequential extraction method enabled the extraction of secondary metabolites from the plant material based on their polarity. It also minimized the antagonistic effect of compounds in the extracts with different polarity (Jeyaseelan *et al.,* 2012). In the present study, dichloromethane VLC fraction showed more activity on the selected bacteria than other VLC fractions. This can be attributed to the presence of bioactive compounds in the extract. The bioactivity of plant extracts is normally influenced by the type of phytochemicals present in the extract. Phytochemicals have been reported to act by various modes of action in carrying out antibacterial activities (Jeyaseelan *et al.*, 2012).

Generally, th0e dichloromethane VLC fraction was more active against the gram-positive bacteria i.e *MRSA* and *S. aureus* than the gram-negative bacteria i.e *P. aeruginosa* and *S. sonnei*. This is because gram-negative bacteria have been reported to be less susceptible to crude extracts than gram-positive bacteria due to the presence of a cell membrane restricting the diffusion of compounds through its lipopolysaccharide layer (Perussi, 2007). The column fractions and isolated compounds as a mixture in the ratio of (1:1:1) also showed some degree of bioactivity against all the selected micro-organisms except *S. sonnei*. This is because *S.sonnie* have been reported to develop resistance to many drugs by extrusion of drugs using active efflux pumps, and overexpression of drug inactivating enzymes (Shahsavan *et al.,* 2017). In contrast to the bioactivity observed in the dichloromethane VLC fraction, the column fractions were more active on gram-negative bacteria *P. aeruginosa* than both gram-positive bacteria. This observation may be attributed to the fact that the amount of the active components in the VLC fraction may have been diluted and fractionation may have increased their concentrations, hence the reason for enhanced bioactivity in *P. aeruginosa*. There could also be the possibility of antagonism among various antibacterial compounds in crude extracts when lumped together thus fractionation may have reduced leading to enhanced activity observed in *P. aeruginosa* (Kuete *et al.,* 2011).

Several studies have reported high bioefficacies of dichloromethane extract in comparison with other crude extracts. A study by Ayepola & Adeniyi (2008) indicated that the dichloromethane fraction exhibited higher activity against *Klebsiella* spp, *Salmonella typhi*, *Yersinia enterocolitica*, and *Bacillus subtilis* (15–16 mm) while the methanol residue had a lower activity against all the test organisms except *Klebsiella* spp and *Salmonella typhi*. The dichloromethane extract of *Ceramium rubrum* showed the highest antibacterial inhibition against the gram-negative bacteria *Yersinia ruckeri* (14.7 mm) and highest antifungal inhibition against *Saprolegnia parasitica* (17.6 mm) in comparison with an ethanolic crude extract which showed inhibition of 8.1mm on *Y. ruckeri* and inhibition of 1.7 mm on *S. parasitica* (Cortés *et al.,* 2014). In another study, the MIC values of ethyl acetate crude extract against *E. coli* and *S. aureus* were 5 mg/ml and 2.5 mg/ml respectively while the MIC for dichloromethane against *E. coli* and *S. aureus* were 2.5 mg/ml and 1.5 mg/ml respectively (Musdja *et al.,* 2019).

Individually, the isolated compounds did not show any bioactivity against the selected microorganisms except jasmolin I which showed some slight activity against *P. aeruginosa*. When the three compounds were mixed at the same concentration and ratio, there was increased bioactivity on the selected micro-organisms suggesting synergy. The activity observed in the compounds as a mixture could be due to cyclopropyl fragment ring in jasmolin I and pyrethrin II. These molecules have been reported to improve the overall activity of the majority of biologically important molecules that contain them by acting as potent alkylation agents (Peterson *et al.,* 2001). The cyclopropyl fragment is a versatile player that frequently appears in preclinical/clinical drug molecules (Talele, 2016). These molecules include quinolone antibiotics such as ciprofloxacin, clinafloxacin, gemifloxacin, and moxifloxacin. Other molecules that have the cyclopropyl fragment include tyrosine kinase inhibitor (4/lucitanib), HCV NS3/4A protease inhibitors, HIV-1 reverse transcriptase inhibitor (Lumacaftor), calcitriol, calcipotriol, amitifadine and etomide pro-drugs among others (Tanaji, 2016). In molecular structure-activity relationship studies of quinolone antibiotics, it is evident that a cyclopropyl at position 1 of these quinolones is the optimal substituents, regardless of the changes made at other sites (Peterson, 2001).

The cyclopropane fragment has been reported to possess a spectrum of biological properties ranging from enzyme inhibitions to insecticidal, antifungal, herbicidal, antimicrobial, antitumor, and antiviral activities. Previous studies have shown that cyclopropane associated with fatty acids has been proven to have antifungal activity (Pohl *et al.,* 2011). Another study showed cyclopropane associated with fatty acids from the marine bacterium *labrenzia* exhibited antimicrobial activity and it activated orphan G-protein coupled receptor GPR84, which is vastly expressed on immune cells (Moghaddam *et al.,* 2018).

The fact that extracts of *C. cinerariaefolium* showed some degree of activity against the selected bacteria is an indication that the plant has a broad spectrum of bioactivity against both gram-negative and gram-positive bacteria. This concurs with a previous study that showed that large numbers of *Chrysanthemum* extracts were active against both gram-positive and gram-negative bacteria (Sassi *et al.,* 2008). It is also coinciding with previous studies that showed that flowers of members of the *Chrysanthemum* genus (*Asteraceae*) possess phytochemicals that are of medicinal importance (Jung, 2009). For example, flowers of *Chrysanthemum indicum* have been used in folk medicine for the treatment of several infectious diseases such as pneumonia, colitis, stomatitis, cancer, fever, soreness, and hypertensive symptoms (Jung, 2009).

5.2 Cytotoxicity of pure compounds as a mixture (1:1:1)

Evaluation of cytotoxicity of plant extracts and compounds is useful in determining the toxicological risks associated with the use of plant-derived compounds for medicinal purposes. According to the guidelines set by the National Cancer Institute (NCI) (Geran *et al.,* 1972), extracts are considered cytotoxic if their IC_{50}< 20 µg/ml. From cytotoxicity results, the compound mixture was considered noncytotoxic against Vero cells since the IC_{50} was 74.45 µg/ml.

Previous toxicity studies on pyrethrins showed the extract had low toxicity on mammals and disintegrate quickly under environmental conditions such as sunlight (Chen and Casida, 1969). In previous study on rats and rabbits, pyrethrins failed to show developmental toxicity in rats or rabbits at the highest maternally toxic doses tested, which were 75 and 250 mg/kg per day, respectively (Schardein, 1987b, 1987d). Rabbits tolerated dermal exposure to 2000 mg/kg. However, mild to well-defined erythema with insignificant edema was observed. All animals seemed normal through the 14-day observation period. Besides, the application of pyrethrins to the skin of albino rabbits produced negligibly irritation on the skin (Romanelli, 1991b).

Metabolism of pyrethrin I has been noted to proceed through oxidative processes, while that of pyrethrins II through a combination of hydrolytic and oxidative processes. The resulting metabolites have been identified not to be of toxicological concern. This follows a study in which the metabolism of the six natural pyrethrins by mouse and rat microsomes was assessed in vitro. This further confirmed the safety of pyrethrins (Class *et al.,* 1989).

5.3 Characterization of isolated secondary metabolites

As mentioned in the previous chapters, three pure compounds were isolated from the flowers of *C. cinerariaefolium* dichloromethane extract. These compounds were jasmoline I (**1**), pyrethrin II (**2**)**,** and cinerolone (**3**). The three isolated compounds belong to the family of pyrethrins, a group of six monoterpenes, and the principal secondary metabolites present in the pyrethrum plant (Essig & Zhao, 2001). They are produced by the esterification of chrysanthemic acid and pyrethric acid. Pyrethrins classified as chrysanthemic acid esters are cinerin I, jasmolin I, and pyrethrin I and are collectively known as the pyrethrin I fraction. Pyrethric acid esters are cinerin II, jasmolin II, and pyrethrin II and are known as pyrethrin II fraction (Essig & Zhao, 2001a). Cinerolone is the keto alcohol moiety of pyrethrins (Matsuda, 2011).

Pyrethrin II has been previously isolated, (Bramwel *et al.,* 1969; Rugutt *et al.,* 1999). Likewise, jasmoline I and Cinerolone has been isolated by Bramwel *et al.,* (1969). Besides *C. cinerariaefolium,* other plants of the genus *Chrysanthemum* such as *Chrysanthemum morifolium* have been reported to contain pyrethrins (Simanjuntak *et al.,* 1998). Plants such as *Calendula officinalis*, *Tagetes erecta*, *Tagetes minuta*, *Zinnia elegans*, *Zinia linnearis* also contain pyrethrins but in fewer amounts (Paramesha *et al.,* 2018).

5.4 Synthesis and characterization of silver nanoparticles

Plant extracts have been used widely in the green synthesis of nanoparticles because they are more stable and the rate of synthesis is faster in comparison to using other natural extracts such as microorganisms and fungi (Srirangam & Rao, 2017). This is because of the simple and one-step method that does not require culturing or purification (Reda *et al.*, 2019). Earlier studies have shown that crude extracts from various plants such as *Malachra capitata*, *alfalfa sprouts*, *Aloe spp*, banana peel, *Annona squamosa* peel extract, bamboo charcoal, *Curcuma longa* tuber, *Eucalyptus hybrida, Cinnamon zeylanicum* bark, *Geranium* spp, and *Capsicum annuum* among many other medicinal plants, have been explored for the synthesis of silver nanoparticles (Iravani, 2011; Rai *et al.*, 2009). Although many nanoparticles have been successfully synthesized using plant extracts, a search for nanoparticles with precise biological, physical, and chemical features is still at the cutting edge of nanoscience research.

In the present study, nanoparticles were synthesized using dichloromethane, dichloromethane-methanol, methanol, dichloromethane-ethyl acetate, ethyl acetate, and aqueous crude extracts of *C. cinerariaefolium* resulting in dichloromethane-Ag NPs, dichloromethane-methanol-Ag NPs, methanol-Ag NPs, dichloromethane-ethyl acetate-Ag NPs, ethyl acetate-Ag NPs, and aqueous-Ag NPs. The synthesis of the nanoparticles was ascertained when a colour change to brown was observed on the addition of aqueous silver nitrate to respective crude extracts. Observation of colour change on the mixture (aqueous silver nitrate and plant extracts) acted as primary notable evidence of the formation of colloidal solution of Ag NPs (Chandran *et al.*, 2006).

The colour change was due to the excitation of Surface Plasmon Resonance (SPR) by Ag NPs, which was detected, in a UV-visible spectrum. The color of colloidal silver nanoparticles is endorsed to surface plasmon resonance (SPR) which arises from the collective oscillation of free conduction electrons caused by an interacting light wave (Kharissova *et al.*, 2013). SPR depends on several parameters, such as size, the shape of the nanoparticles, and the medium in which the particles are suspended (Ashour *et al.*, 2015).

In the current study, the UV-Vis spectra showed SPR peaks at 434 nm, 439 nm, 430 nm, 445 nm, 446 nm, and 449 nm belonging to dichloromethane-Ag NPs, aqueous-Ag NPs, dichloromethane-methanol-Ag NPs, methanol-Ag NPs, dichloromethane-ethyl acetate-Ag NPs, and ethyl acetate-Ag NPs respectively. As observed in the current study, earlier studies have also confirmed that the SPR peak for silver nanoparticles is located within 410-460 nm regions (Megiel, 2017). Another absorbance peak at around 325 nm in aqueous-Ag NPs and dichloromethane-Ag NPs could be attributed to the absorbance of *C. cinerarifolium* moieties. This is because the peak has also been observed in the UV-Vis of all the plant extracts used in the current study. Moreover, a similar peak has been observed in other studies when plant extracts were used in the synthesis. A localized peak between 300 nm and 380 nm wavelength related to *B. globosa* extract has been observed in silver nanoparticles synthesized using the extract (Carmonaa *et al.,* 2017). The presence of another peak around 360 nm attributed to onion moieties have been reported in the synthesis of silver nanoparticles using onion (Hussein *et al.,* 2019). No prominent peak was observed at 410-460 nm regions in the UV-Vis spectrum of silver nitrate and plant extracts an indication of the absence of SPR band. This corroborated with previous studies (Ashraf *et al.,* 2020). High SPR peak intensity in the case of aqueous-Ag NPs, ethyl acetate-Ag NPs, and methanol-Ag NPs suggest the relatively stronger reducing power in these extracts used in the biosynthesis of the nanoparticles (Mahidul *et al.*, 2019).

One plasmonic resonance was observed in the UV-Vis spectra of all the silver nanoparticles attributable to the symmetry of the particles. This property depicts that the nanoparticles were spherical to nearly spherical (Noguez *et al.*, 2007). This is in agreement with what was observed in TEM and SEM micrographs of all the nanoparticles, which indicated that the particles were spherical to nearly spherical. Numerous sizes of spherical Ag NPs synthesized using bio-organic compounds have been reported in previous studies as determined by TEM and SEM analysis (Aziz *et al.*, 2019; Elbeshehy *et al.*, 2015; Ghiuţă *et al.,* 2017; Mahmoud *et al.,* 2016; Oves *et al.,* 2018;). The broader peaks in the UV-Vis spectra signified increased polydispersity that is attributed to variations in the growth rates of individual particles during the nucleation step (von White *et al.*, 2012). Varying sizes of the Ag NPs were also observed in TEM and SEM micrographs.

The average sizes of the nanoparticles from the histograms generated from measuring the diameters of the TEM micrographs were 27±12.2 nm, 24.4±8.8 nm, 22.8±17.5 nm, 31.8±11.4 nm 34.7±20.3 and 75.3± 19.7 nm for dichloromethane-methanol-Ag NPs, aqueous-Ag NPs, dichloromethane-Ag NPs, methanol-Ag NPs, dichloromethane-ethylacetate-Ag NPs, and ethyl acetate-Ag NPs respectively. This agrees with the UV-Vis spectra, which showed that smaller nanospheres have their absorbance peak near 400 nm while larger nanospheres had their absorbance peak shift towards longer wavelengths (Paramelle *et al.,* 2014). The absorption maxima of Ag NPs shifting to a longer wavelength in UV-vis spectra with an increase in nanoparticle size has previously been reported (Agnihotria *et al.,* 2014). A previous study showed a decrease in particle size when a blue shift (from 455 to 436 nm) was observed in the synthesis of Ag NPs using *Tulsi* extract and from (429 to 405 nm) in the case of synthesizing the nanoparticles using quercetin which both indicated decreased particle size (Saware *et al.,* 2014). With an increase in the diameter of the particle, the energy required to excite the surface plasmon electrons decreases. As a result, the absorption maximum shifts towards longer a wavelength (Jain *et al.,* 2017).

From the study, those nanoparticles that were bigger e.g ethyl acetate-Ag NPs (75.3± 19.7 nm) could have been formed due to the high concentration of the bio-reducing agents in the ethyl acetate extract, leading to very small nanoparticles that tend to aggregate and result in the formation of larger stable particles (Von White *et al.,* 2012). Aggregation affects the optical properties of silver nanoparticles causing the surface plasmon resonance to shift to lower energies by red-shifting to longer wavelengths (https://nanocomposix.com/pages/silver-nanoparticles-optical-properties). Aggregation of nanoparticles was also observed in TEM and SEM micrographs.

Reduction of silver nitrate ions (Ag^+) to Ag^0 took varying duration depending on the extract. Some took short (48 hours) i.e dichloromethane-Ag NPs, dichloromethane-ethyl acetate-Ag NPs, methanol-Ag NPs, and aqueous-Ag NPs while others took a long duration (96 hours) i.e ethyl acetate-Ag NPs and dichloromethane-ethyl acetate-Ag NPs. The duration was ascertained since, after the respective time, the resonance plasmon absorption peak remained unaffected, which confirmed the bio-process was completed. The difference in duration could be attributed to the concentration of the reducing agents in the respective extracts. The reaction time has been noted to vary depending on the concentration of active ingredients, type of plant extracts, reaction temperatures, and pH (Ahmed & Mustafa, 2020). Different plant extracts have been reported to take varying times in the reduction of Ag ions to Ag nanoparticles. Some have been reported to take between 24 and 72 hours, or even a week (Dipankar & Murugan, 2012, Khalil *et al.,* 2014).

The elemental constituents of Ag NPs were determined using Energy Dispersive X-ray (EDX). The spectrum showed a strong signal at the silver region i.e around 3-3.7 Kev in all the nanoparticles. This is in agreement with other studies whereby metallic silver nanoparticles have been reported to show typical optical absorption peaks at approximately 3.7 Kev (Khan *et al.,* 2016). A strong signal for Ag is an indication that the Ag NPs were successfully formed by the flower extracts of *C. cinerariaefolium* (Gopinath *et al.,* 2015). The other EDX peaks assigned to carbon and oxygen imply that plant constituents successfully capped the nanoparticles (Dada *et al.,* 2017d).

The presence of other peaks in EDX analysis belonging to other elements such as carbon and oxygen have been observed in other studies where plant extracts have been used to synthesize the metallic nanoparticles (Khan *et al.,* 2016). Nonetheless, no signal of N from Ag NO_3 was observed in all the synthesized Ag NPs. This corroborates with a study done by (Vorobyova *et al.,* 2020) who also observed the absence of nitrogen signal in the EDX. The percentage of Ag metal in occurrence with other chemical elements varied with extracts. Dichloromethane-methanol-Ag NPs had the highest percentage of silver at (81.33%), followed by ethyl acetate-Ag NPs (72.19%), dichloromethane-ethyl acetate (69.54%), dichloromethane (67.26%), and methanol (56.58%). Aqueous extract was least with (14.01%). A significant percentage of silver (greater than 50%) in the current study has been observed in other studies. A study done by Srirangam & Rao (2017) indicted that silver had a percentage of (70.36%). Nonetheless, other studies have also reported percentage of silver to be less than 20% similar to what was observed in aqueous extract (Ponarulselvam *et al.,* 2012).

FTIR analysis was carried out to determine the functional groups in various extracts that were present on the surface of the formed nanoparticles. The functional groups were responsible for the reduction of the silver ions (Ag^+) to Ag NPs (Ag^0) and the stabilization. In the study, absorption peaks observed in the range of (3200-3600 cm^{-1}) in all synthesized silver nanoparticles were assigned to the stretching vibration of O-H groups of phenolic compounds (Yulizar & Hafizah, 2015). The bands at 2300-2400 cm^{-1} in dichloromethane-Ag NPs, methanol-Ag NPs, dichloromethane-ethyl acetate-Ag NPs, and ethyl acetate-Ag NPs were attributed to the presence of C=O band (Dorranian *et al.,* 2012). The absorption bands in the range of (1620–1680 cm^{-1}) in all the synthesized Ag NPs corresponded to the C=C of aromatic compounds of the phytochemicals (Raghunandan *et al.,* 2010).

The C-O stretching vibration for the carbonate group appeared at wave number 1400-1600 cm^{-1} (Meejo *et al.,* 2006). The peaks in the range of 1000-1150 cm^{-1} were assigned to the carboxylic acid stretching bands (Correia *et al.,* 2016). Bands with wave numbers around 615-437 cm^{-1} have been ascribed to intrinsic stretching vibrations of the metal-oxygen bond (Tripathi *et al.,* 2011). The identified functional groups in the FTIR analysis belong to phytochemicals present in the extracts. Plant phytochemicals have been reported to play a crucial role in the synthesis of nanoparticles since they act as reducing and capping/stabilizing agents in the reduction of metal ions to metal nanoparticles (Swarnalatha *et al.,* 2013).

The identified phytochemicals in the present study have been reported to reduce the silver ion and stabilize silver nanoparticles. The major phytochemicals that have been implicated in the synthesis of metal nanoparticles are flavonoids and phenolic compounds due to the presence of hydroxyl groups and carbonyl moieties (Marslin *et al.,* 2018). For example, quercetin, a flavonoid noted to have an extended system of conjugated double bonds and five hydroxyl groups gave high reductive ability on silver ions forming silver nanoparticles (Terenteva *et al.,* 2015). Catechol on the other hand having two hydroxyl groups in ortho positions have been reported to form a stable complex with metal cations such as Mo(VI), Fe(II)/Fe(III), Cu(II), Zn(II), Al(III), Tb(III), Pb(II), Co(II) (Cherrak *et al.,* 2016). Green synthesis of Ag NPs using both leaf extract of *Ocimum sanctum* and a flavonoid (quercetin) was present in the extract (Jain & Mehata, 2017).

Tannins present in oak bark extracts were reported to be responsible for the reduction of silver nanoparticles. Although tannin acid is identified as a weak reducer and forms germs of nanoparticles, the reduction of silver ions may be possible because of products of tannic acid hydrolysis, which are glucose and gallic acid (Puišo *et al.,* 2012a). Garlic and ginger extracts have been reported to contain large amounts of flavonoids and phenolic compounds, which have an important role in the reduction process during the synthesis of metal nanoparticles (El-Refai *et al.,* 2018).

Tulsi plants have been reported to have flavonoids, terpenoids, and phenolic compounds that were responsible for the reduction of silver ions to Ag NPs (Jain & Mehata, 2017). The exact mode of action of flavonoids in reducing silver ions to nanoparticles is unknown but it might be possible that the tautomeric transformation of flavonoids from enol form to keto form could release reactive hydrogen atom that reduces silver ions to silver nanoparticles (Jain & Mehata, 2017).

Flavonoids, glycosides, and carbohydrates in *Lantana camara* were responsible for the synthesis of silver nanoparticles (Ajitha *et al.,* 2015). The FTIR and phytochemicals analysis revealed the multifunctionality of *C. cinerariaefolium* extracts in carrying out reduction and stabilization of silver nanoparticles.

5.5 Antibacterial activity of synthesized Ag NPs

Antibiotics resistance is a threat to humanity hence this has necessitated a search for alternative novel drugs to fill the gap (Ayaz *et al.,* 2019). Silver inform of silver ions have been used widely due to their antibacterial properties (Lok *et al.,* 2006). The immense antibacterial property in silver ions is attributed to the high tendency of silver to sulfur and phosphorus molecules. These two components i.e sulfur and phosphorus are found abundantly throughout the membrane of the bacterial cell. Silver ions, therefore, react with proteins containing sulfur inside or outside of the cell membrane hence affecting cell survival (Tamboli *et al.*, 2013).

The application of nanotechnology in the synthesis of silver nanoparticles has improved immensely the antimicrobial activity of silver (Huh *et al.,* 2011). Overall, in the present study, the antibacterial activity of Ag NPs was higher than the antibacterial activity of silver ions and plant extracts at the same contration an indication that the silver nanoparticles exhibited good antimicrobial activity against selected pathogenic bacteria. This observation has been observed in previous studies (Khan *et al.,* 2016). Ethyl acetate-Ag NPs that were the largest nanoparticles 75.3±19.7 nm showed the least inhibition on all the selected bacteria. This is in agreement with previous studies, which showed that the inhibitory effect of silver nanoparticles normally depends on particle size (Puišo *et al.,* 2014). Many studies have reported small-sized Ag NPs do possess the best antibacterial activity compared to large nanoparticles. For example, a better antibacterial activity was observed when using 5-nm Ag NPs compared with other sized Ag NPs (10, 15, and 20 nm) Choi & Hu (2008). The highest antibacterial activity was also observed with 8.4 nm Ag NPs compared with three different sizes (8.4, 16.1, and 98 nm) against *Staphylococcus mutans* (Espinosa-Cristobal *et al.*, 2009). Another study showed 89 nm Ag NPs had a MIC of 33.71 µg/ml compared to 7.5 µg/ml for 7-nm Ag NPs against *S. aureus* (Martı́nez-Castañ̃ón *et al.*, 2008). Small size Ag NPs have been reported to have the best antibacterial properties since they easily reach the nuclear content of bacteria and they present the greatest surface area that is in contact with bacteria (Lok *et al.*, 2006).

From the results, all synthesized silver nanoparticles did not show any significant difference in the activity against either both gram-negative and gram-positive bacteria or one gram-negative and gram-positive bacterium. Previous studies provide conflicting statements on the effect of nanoparticles on gram-positive and gram-negative bacteria. Gram-negative bacteria are more resistant to the effect of silver nanoparticles (Shrivastava *et al.,* 2007) while silver nanoparticles have an equal inhibitory effect against both gram-positive and gram-negative strains (Kong & Jang 2008; Peticae *et al.,* 2008). The ability of Ag NPs to act on both gram-negative and gram-positive bacteria has been ascertained in previous studies therefore the present study confirms the multifaceted strategy of Ag NPs (Franci *et al.,* 2015). From the study, those nanoparticles that caused an inhibition of 10 mm and above were subjected to MIC. The MIC of dichloromethane-methanol- Ag NPs determined against

S.aureus was 31.25 µg/ml. This corroborates a previous study whereby the green synthesized Ag NPs using *A. reticulata* extract also had a MIC of 31.25 µg/ml against *S. aureus* (Shrivastava *et al.,* 2007). The MIC of dichloromethane-Ag NPs against *P. aeruginosa* was 15.625 µg/ml. Previous studies also have reported a MIC of 15.63 µg/ml when lipopeptide stabilized Ag NPs was subjected to *P. aeruginosa* (Bezza *et al.*, 2020).

5.6 Cytotoxicity studies of synthesized Ag NPs and extracts used in the synthesis

Nanotechnology has been embraced by industrial sectors and in medicine due to the remarkable potential applications of nanoparticles and nanomaterials. The advance in nanotechnology and the use of nanoparticles presently, has elicited a growing public debate on the toxicity and environmental impact of direct and indirect exposures to nanoparticles (Brayner, 2008; Panda *et al.,* 2011). According to the US National Cancer Institute (NCI), a substance is considered cytotoxic if the IC_{50} < 20 µg/ml (Nathyadevi and Sivakumar, 2015). In the current study, none of the tested plant extracts and biosynthesized Ag NPs had an IC_{50} < 20 µg/ml, hence were not considered cytotoxic. This agrees with a previous study that showed that Ag NPs in the range between 5 and 45 nm were not significantly toxic against Vero cells at all concentrations tested (Kasithevar *et al.,* 2017).

In the current study, small-sized nanoparticles i.e dichloromethane-Ag NPs had an IC_{50} of 33.33 µg/ml while the largest nanoparticles ethyl acetate-Ag NPs had an IC_{50} of 79.60 µg/ml. This agrees with previous studies that showed that the toxicity of nanoparticles depends on particle size. Small size Ag NPs have been reported to have smaller LD_{50} or IC_{50} values, which translates to stronger cytotoxicity (Pan *et al.,* 2007). There is a tendency for small silver nanoparticles to induce higher reactivity and thus higher genotoxicity (Kim *et al.,* 2011).

The fact that the synthesized nanoparticles were spherical could have aided in reducing the toxicity of the particles since spherical nanoparticles have been reported to be less toxic than other shapes. This is because other shapes such as the wires, hexagonal, etc have been reported to directly contact the cell surface rather than being internalized as in spherical particles making them more toxic than spherical (Stoehr *et al.,* 2011). In a study in which silver nanowires with a diameter of 100-160 nm and length of 1.5-25 µm together with spherical Ag NPs 30 nm in size, were subjected to human lung epithelial A549 cells, spherical particles did not show adverse effects on cytotoxic parameters in A549 cells whereas wires induced negative outcomes (Stoehr *et al.,* 2011).

Although nanoparticle size and morphology have considerable effects on the cytotoxic potential of nanoparticles, other parameters have been reported to be important as well. These parameters include aggregation/agglomeration state, solubility, and surface properties such as attached functional groups, and surface area. They play important roles in influencing the resultant pharmacokinetics and pharmacodynamics of nanoparticles (Yakop *et al.,* 2018).

The Vero cell cytotoxicity of the nanoparticles and extracts was found to be concentration-dependent with the highest concentrations (100 µg/ml) causing decreased cell growth. This has also been observed in previous studies (Prasannaraj & Venkatachalam, 2017).

The cytotoxicity studies showed that silver ions in the form of silver nitrates had an IC_{50} of 21.34 µg/ml, followed by Ag NPs which had an IC_{50} ranging from 33.33 µg/ml-79.80 µg/ml while the plant extracts had the highest IC_{50} (94.01 µg/ml and above). Increased IC_{50} translates to lower toxicity. Lower toxicity of silver nanoparticles compared with silver ions has also been observed in previous studies (Gaiser *et al.,* 2012; Kim *et al.,* 2011, Panda *et al.,* 2011). To investigate cellular death, cultured cells of *Allium cepa* were incubated with silver ions (Ag^+), silver complexes (Ag Cl), capped (Ag NP-P), and uncapped (Ag NP-S) silver nanoparticles. The authors reported that the induction of cell death followed the order Ag^+ ions>colloidal Ag Cl> uncapped Ag NP-Sigma> capped Ag NP-P (biogenic) (Panda *et al.,* 2011).

In comparison to chemically synthesized Ag NPs, green synthesized Ag NPs have been reported to be less toxic. Silver nanoparticles synthesized using ethanolic extract showed 96% of HaCaT cell viability (Senthila *et al.,* 2017) while chemically synthesized Ag NPs showed a 30% viability towards the epithelium cell lines (Gurunathan, 2014). Mangrove fabricated Ag NPs had an IC_{50} of 18.79 ± 0.9l g/ml while chemically synthesized silver nanoparticles produced the same effect (i.e. 50 % cell death) at 8.96 ± 0.8l g/ml (Kumar *et al.,* 2016). Silver nanoparticles synthesized using walnut green husk caused maximum cell death of 15% detected for L-929 fibroblast cells (normal cell line) after 48-hour exposure while commercial silver nanoparticles resulted in 60% cytotoxicity towards the same normal cell line (Khorrami *et al.,* 2018).

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

i. Three compounds were isolated from pyrethrum plant and their structures determined using combination of 1D and 2D nuclear magnetic resonance (NMR) spectroscopic techniques.
ii. The isolated compounds as mixture were more active on *P. aeruginosa* with MIC of 25 gm/mL hence could be used as lead compounds for development of drugs against the bacteria.
iii. Phytochemicals present in the crude extracts of pyrethrum plant successfully synthesized Ag NPs. The plant therefore has the potential to be utilized in green synthesis of nanoparticles.
iv. The silver nanoparticles were active against all the bacteria and indication that silver nanoparticles possess antibacterial activity against both gram positive and gram negative bacteria. As a result, they can also be used as lead compounds in drug discovery against bacteria.
v. Pyrethrum extracts and isolated compounds as a mixture were not toxic against Vero cells, thus are considered safe for utilization in drug discovery.

6.2 Recommendations

i. The isolated compounds both individually and as a mixture should be tested against other microorganisms.
ii. Other plants should be exploited for green synthesis of nanoparticles.
iii. Invivo studies should be carried out to further ascertain the toxicity of Ag NPs

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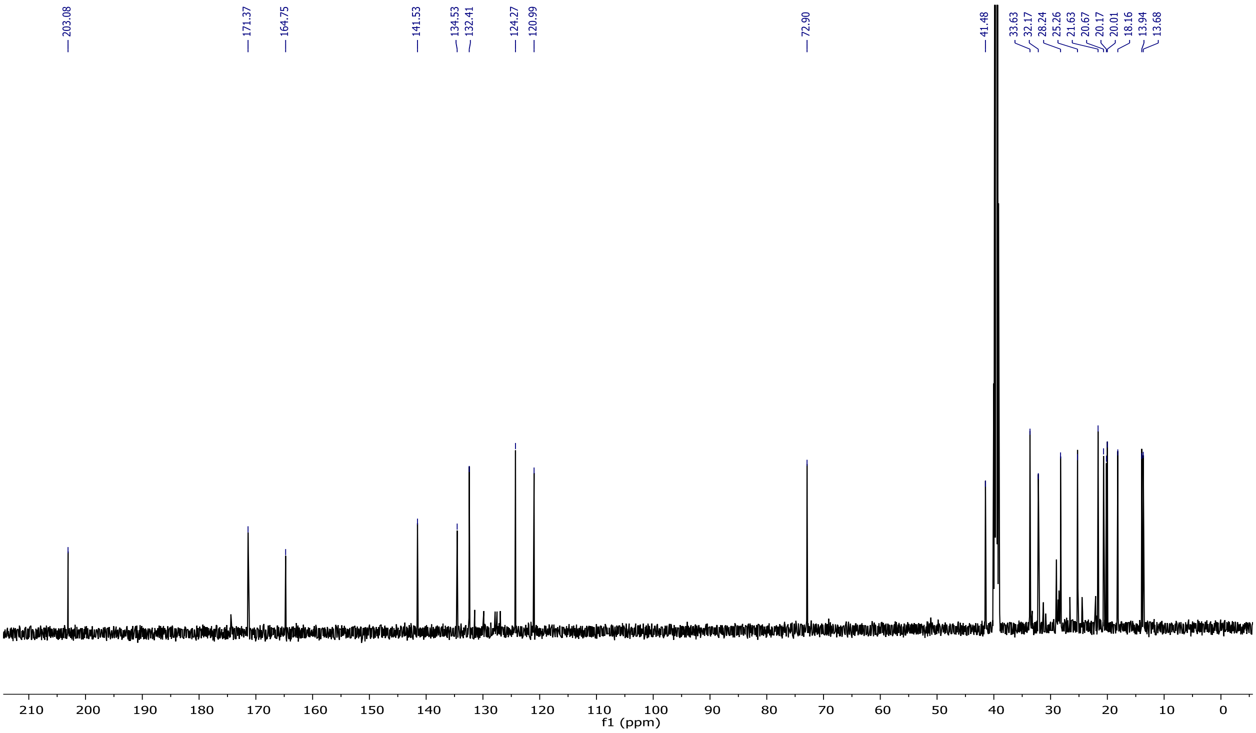
APPENDICES

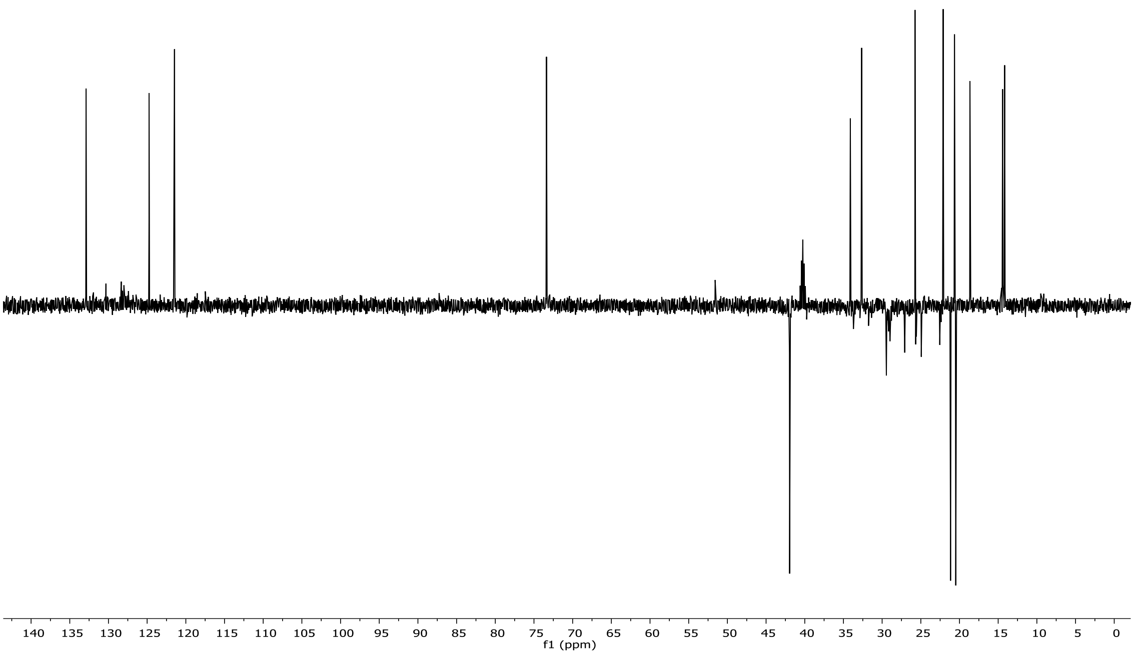
Appendix 1: Preliminary Screening of C. cinerariaefolium VLC Fractions

Extracts	Zone of inhibitions of test organisms in mm			
	MRSA	*S. aureus*	*P.aeruginosa*	*S.sonnie*
Hexane	6 6 6	8 9 8	6 6 6	6 6 6
Dichlromethane-Hexane	10 8 8	9 7 9	6 6 6	6 6 6
Dichloromethane	12 10 10	11 10 10	7 7.5 7	6.5 6 6.5
Dichloromethane-Ethyl acetate	10 10 9	9 7 7	6 6 6	6 6 6
Ethyl acetate	11 9.5 9	6 6 6	6 6 6	6 6 6
Methanol	6 6 6	6 6 6	6 6 6	6 6 6
Dichloromethane-methanol	6 8 7.5	7.8 5.5 7	6 7 6	6 6 6
Positive control	26 28 26	24 23.5 25	25 26 27	23.5 24.5 26
Negative control	6 6 6	6 6 6	6 6 6	6 6 6

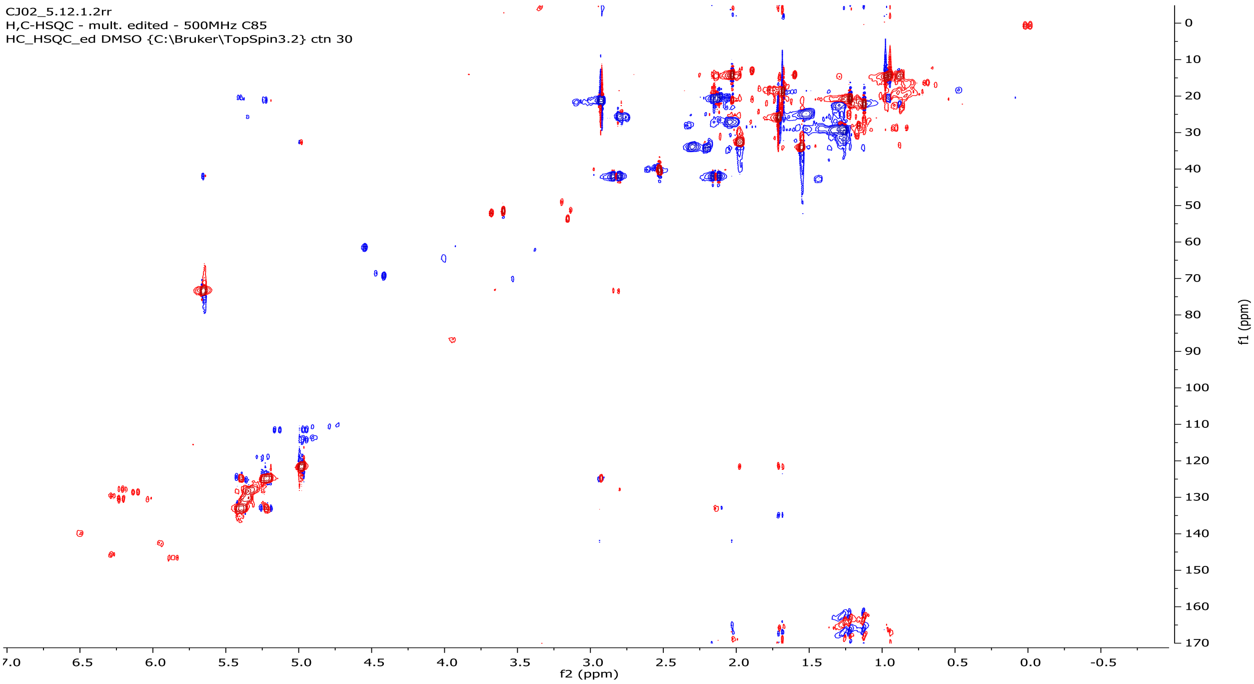
Appendix 2: ^{13}CNMR spectrum for Compound 1



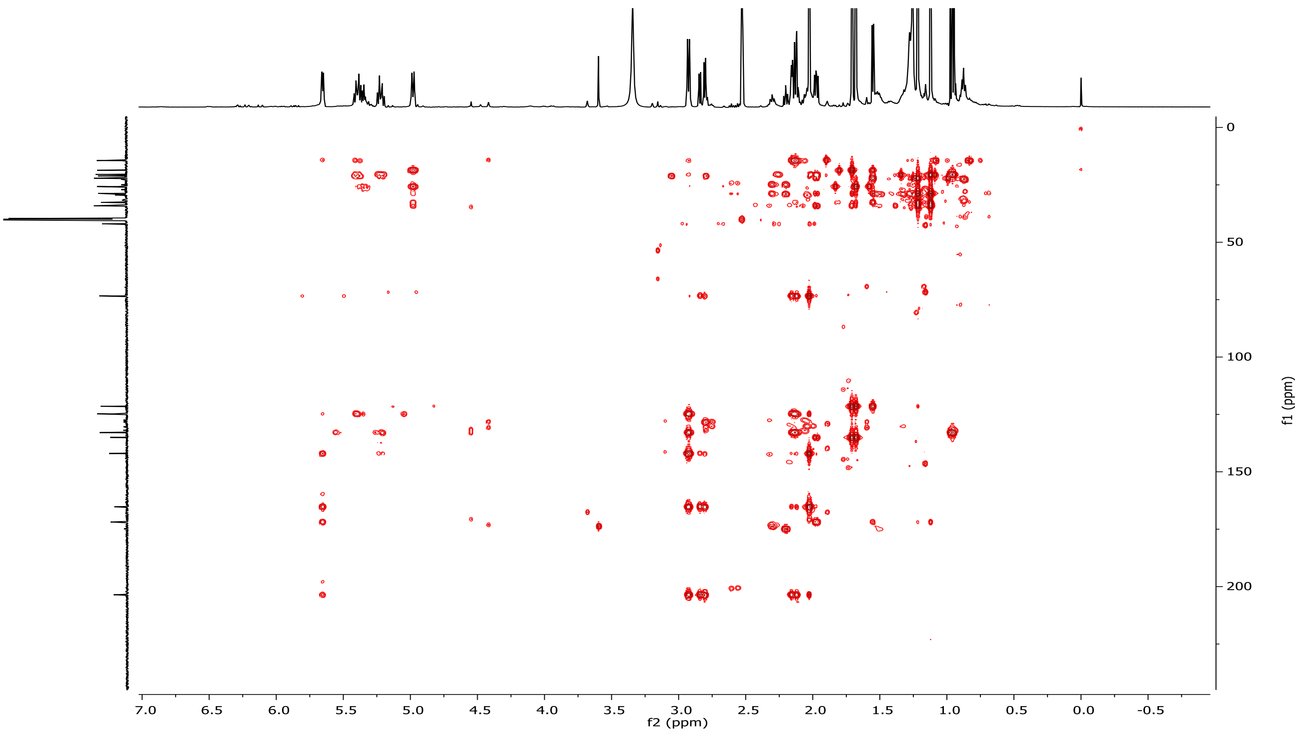
Appendix 3: DEPT spectrum compound 1



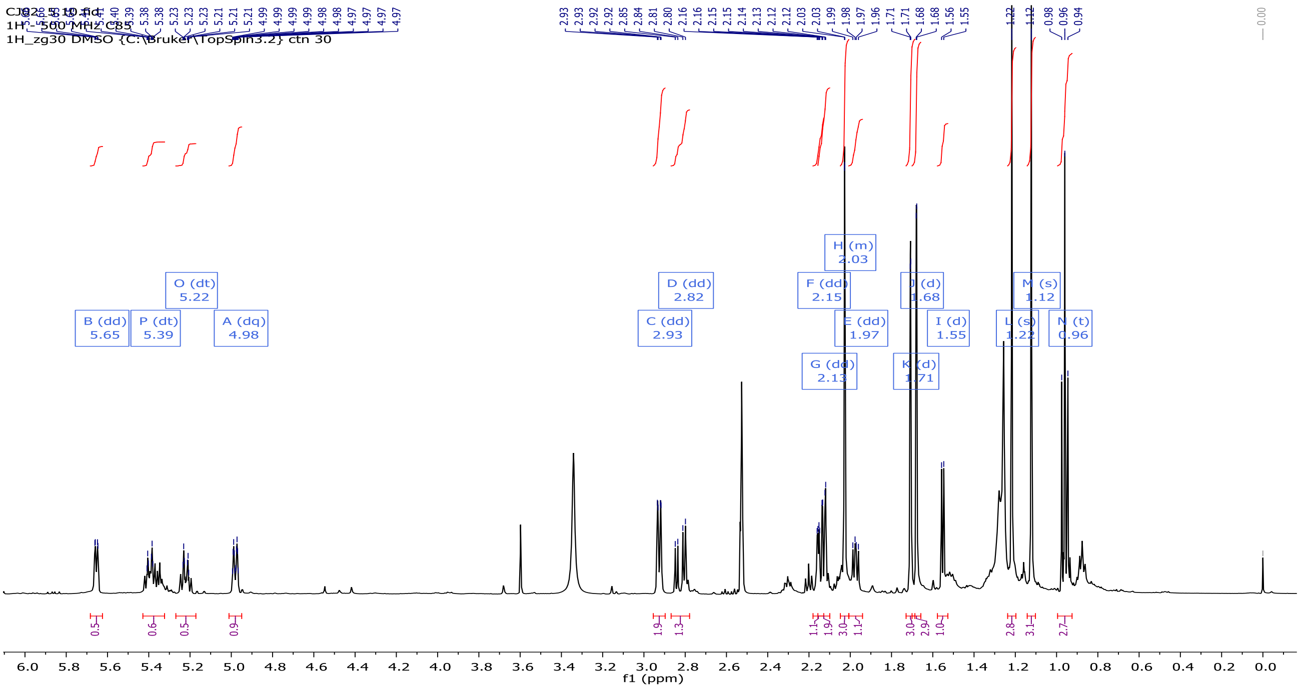
Appendix 4: HSQC spectrum for Compound 1



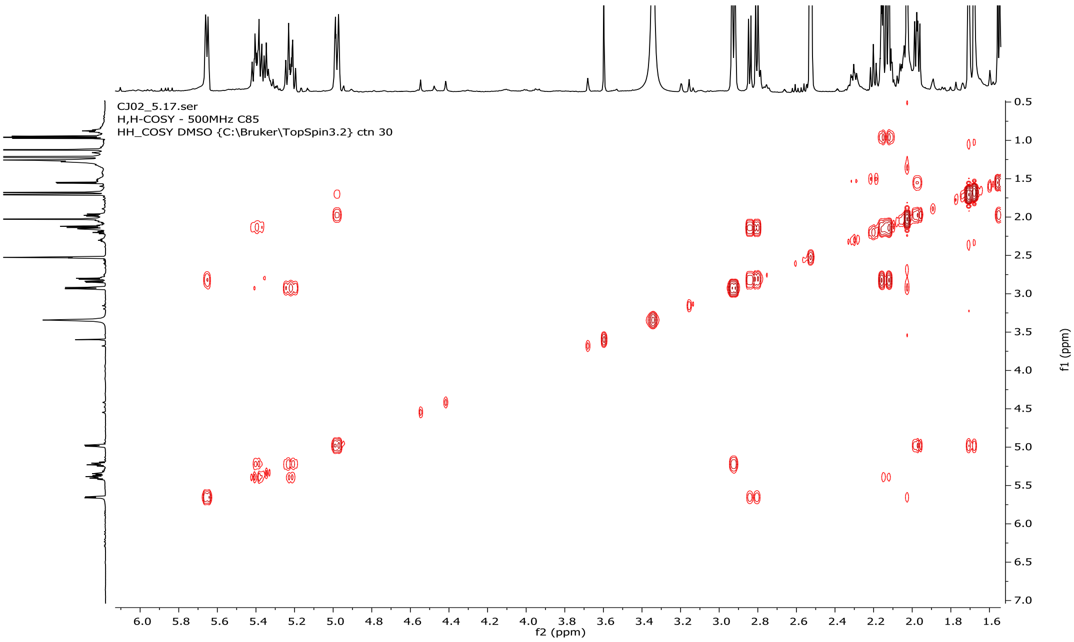
Appendix 5: HMBC compound 1



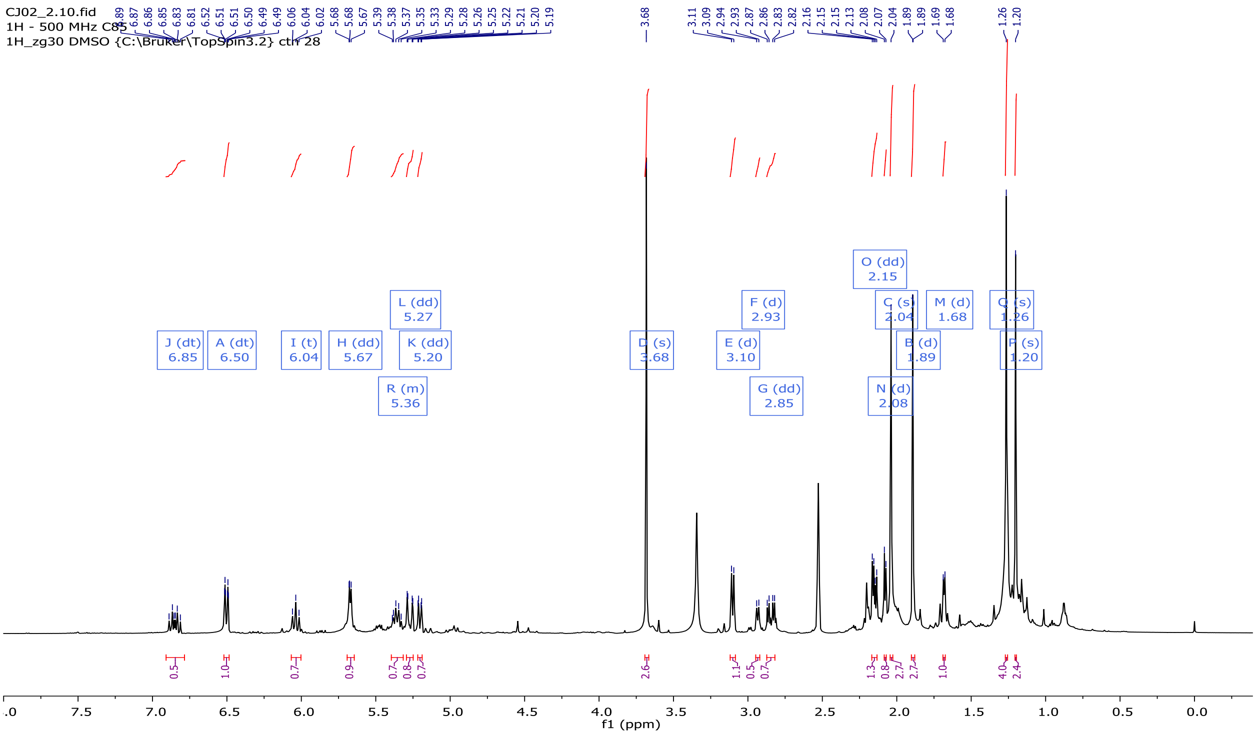
Appendix 5: ^{1}H-NMR spectrum for compound 1



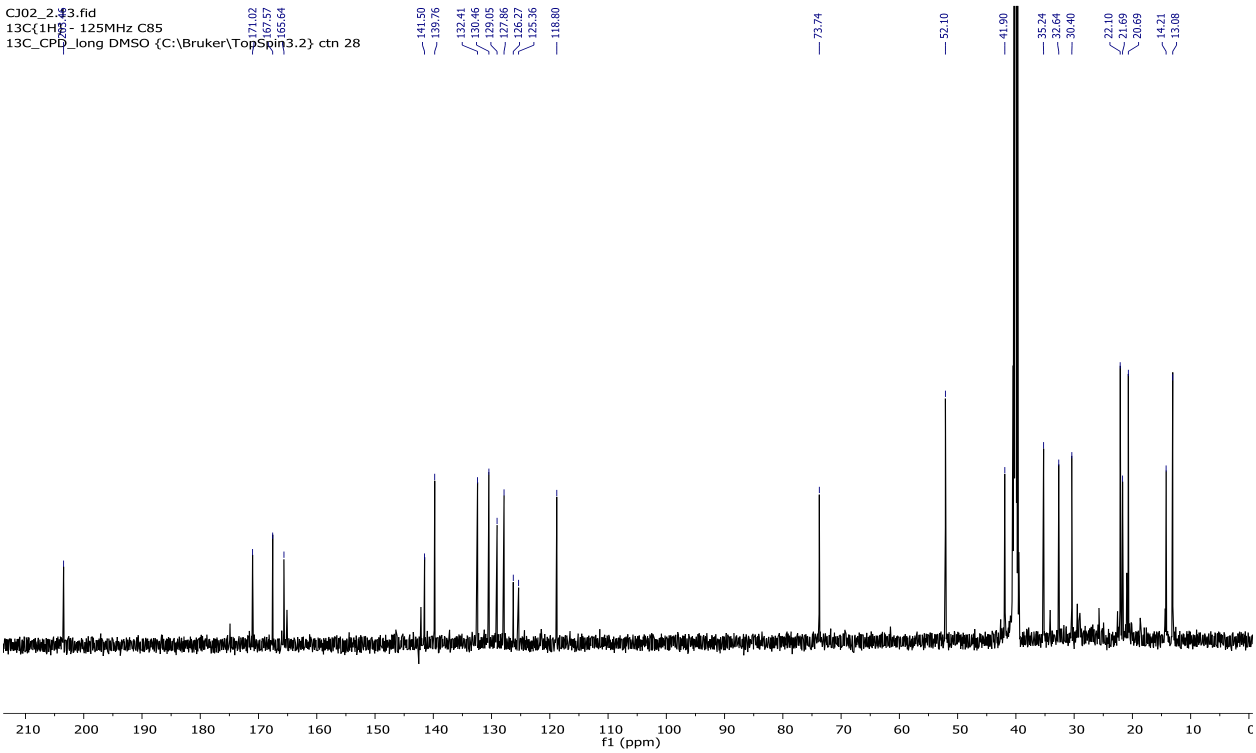
Appendix 6: 1H-1H COSY spectrum for Compound 1



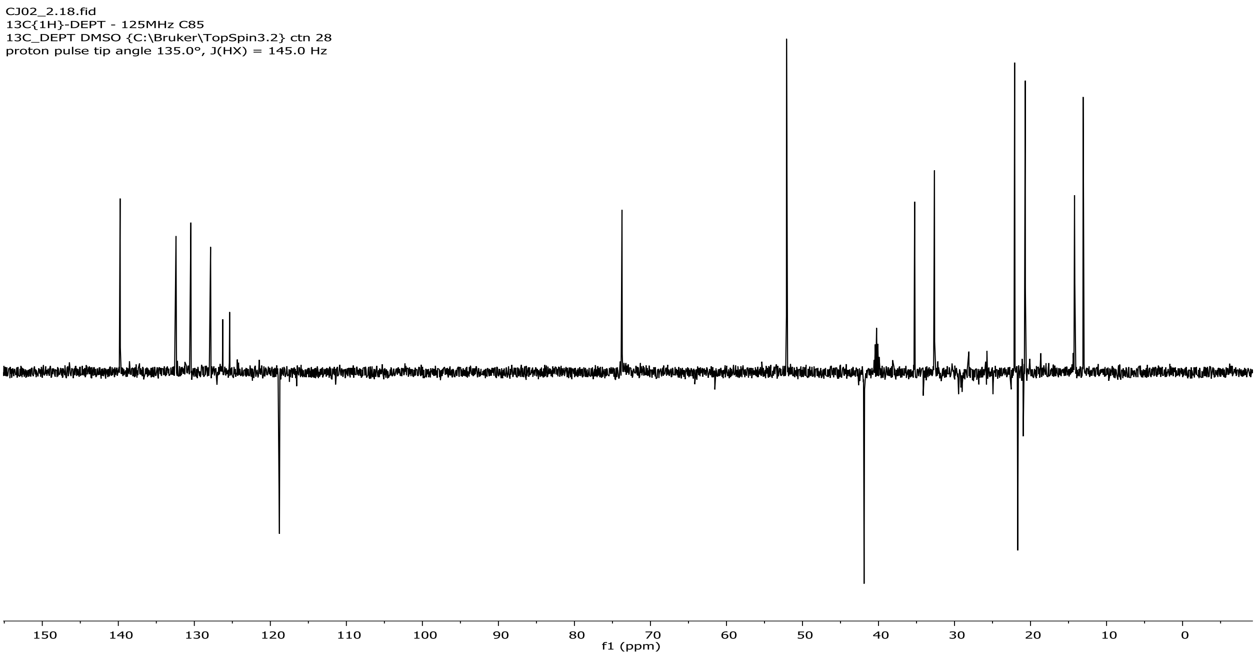
Appendix 7: ¹HNMR spectrum for compound 2



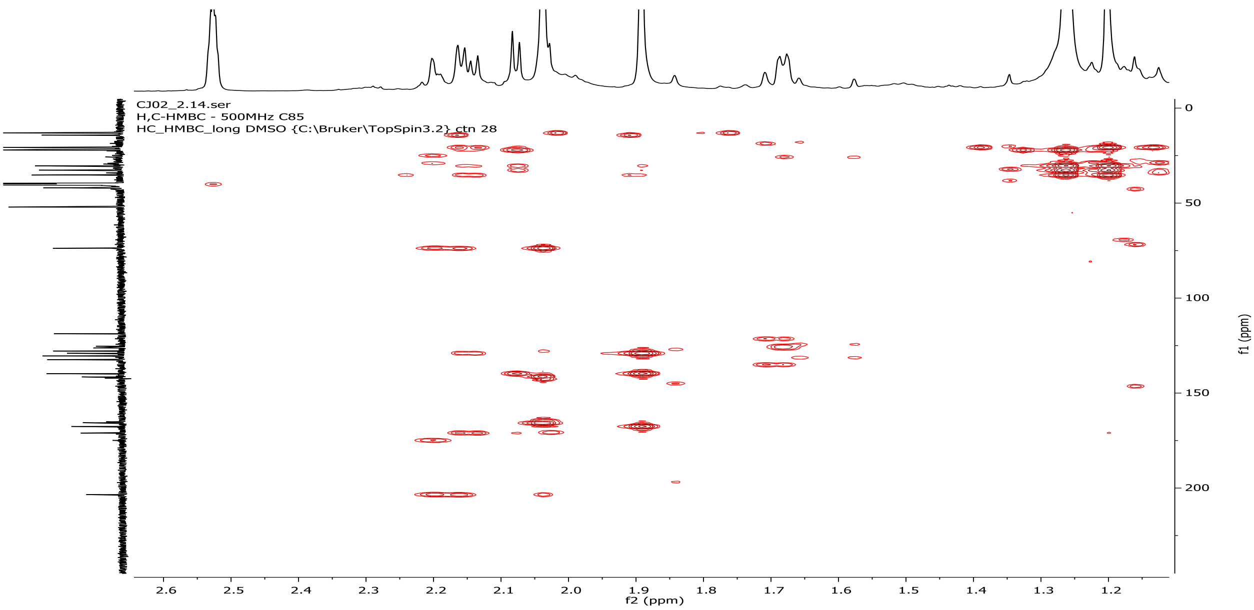
Appendix 8: ¹³C-NMR spectrum for compound 2



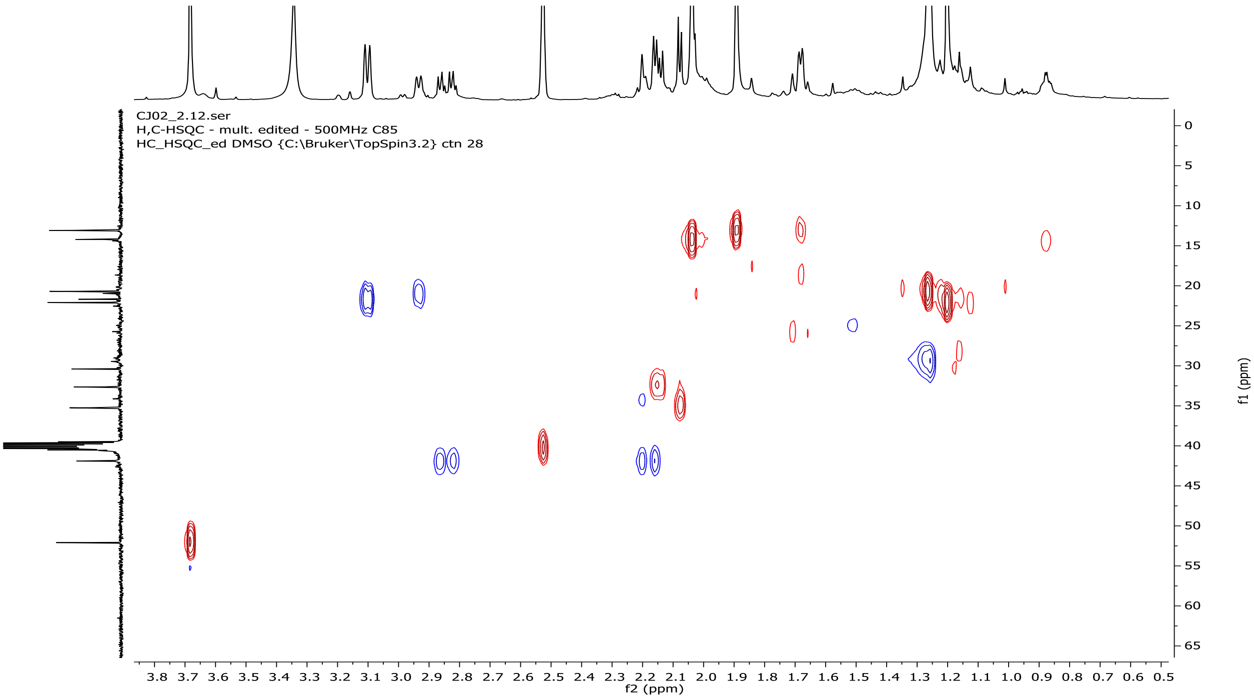
Appendix 9: DEPT spectrum for compound 2



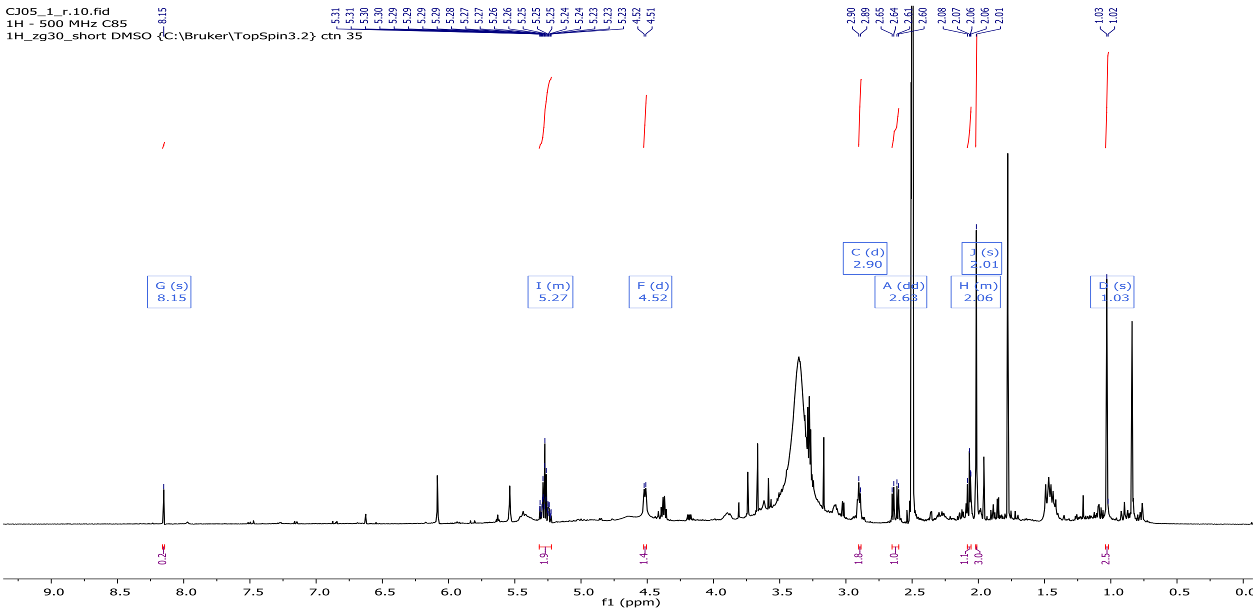
Appendix 10: HMBC spectrum for compound 2



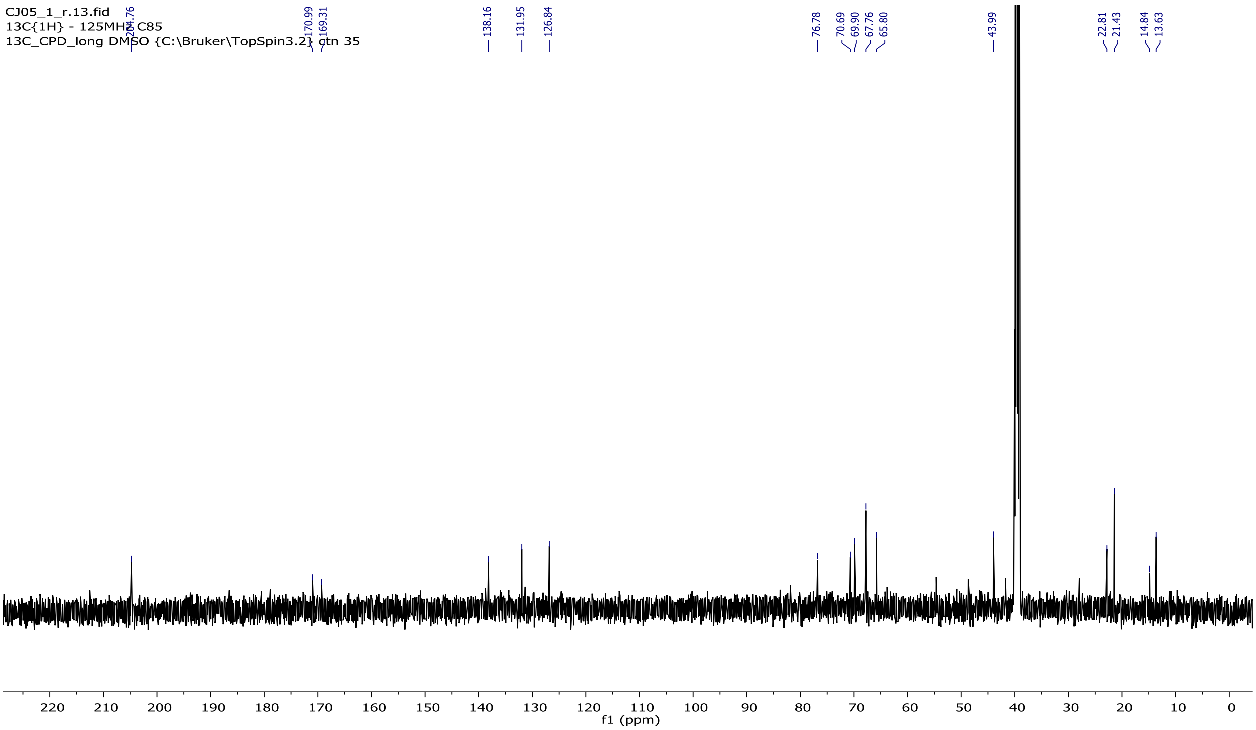
Appendix 11: HSQC for compound 2



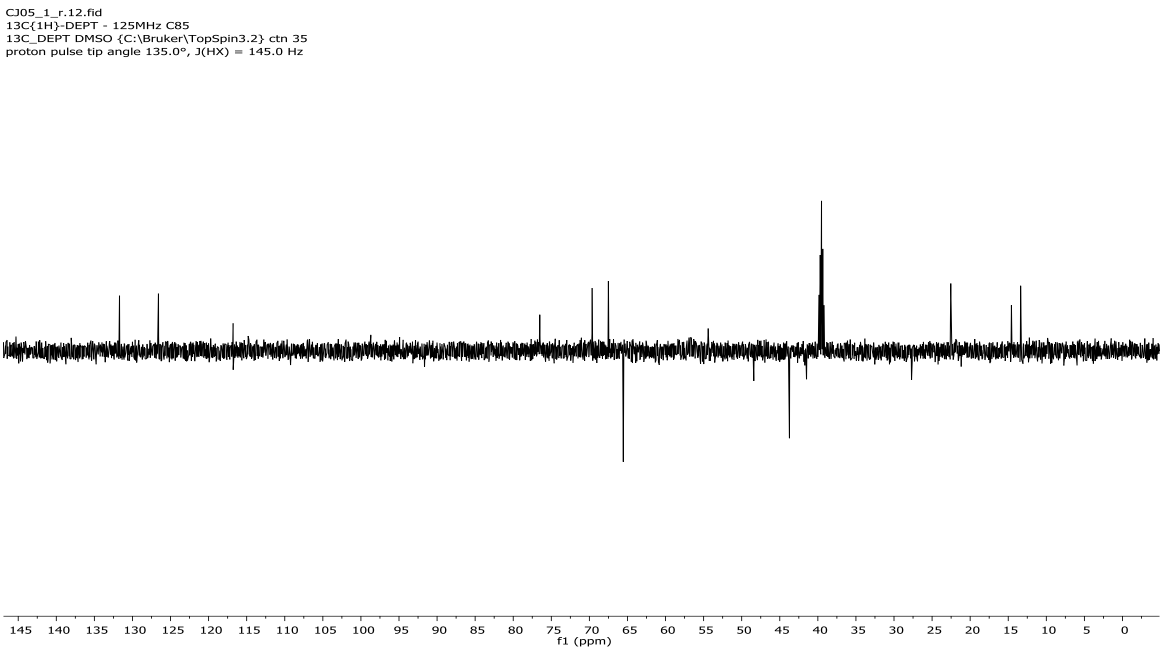
Appendix 12: [1]H-NMR spectrum for compound 3



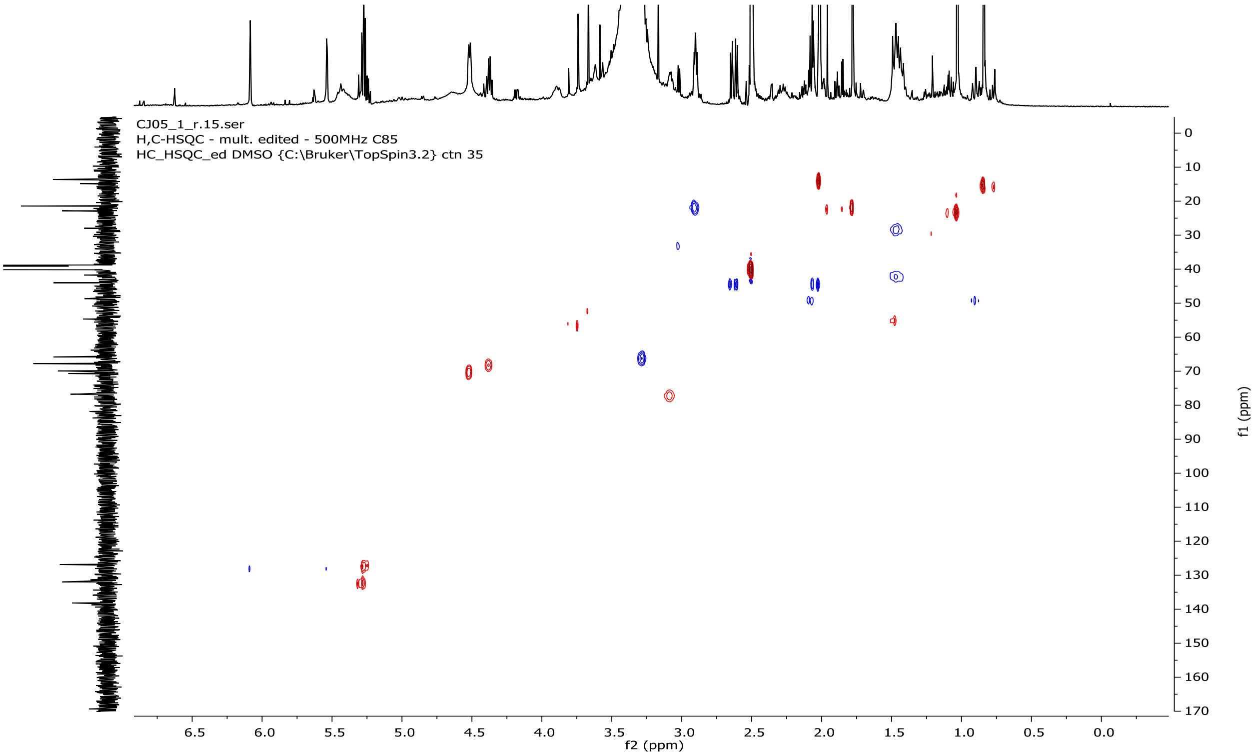
Appendix 13: ^{13}C-NMR spectrum for compound 3



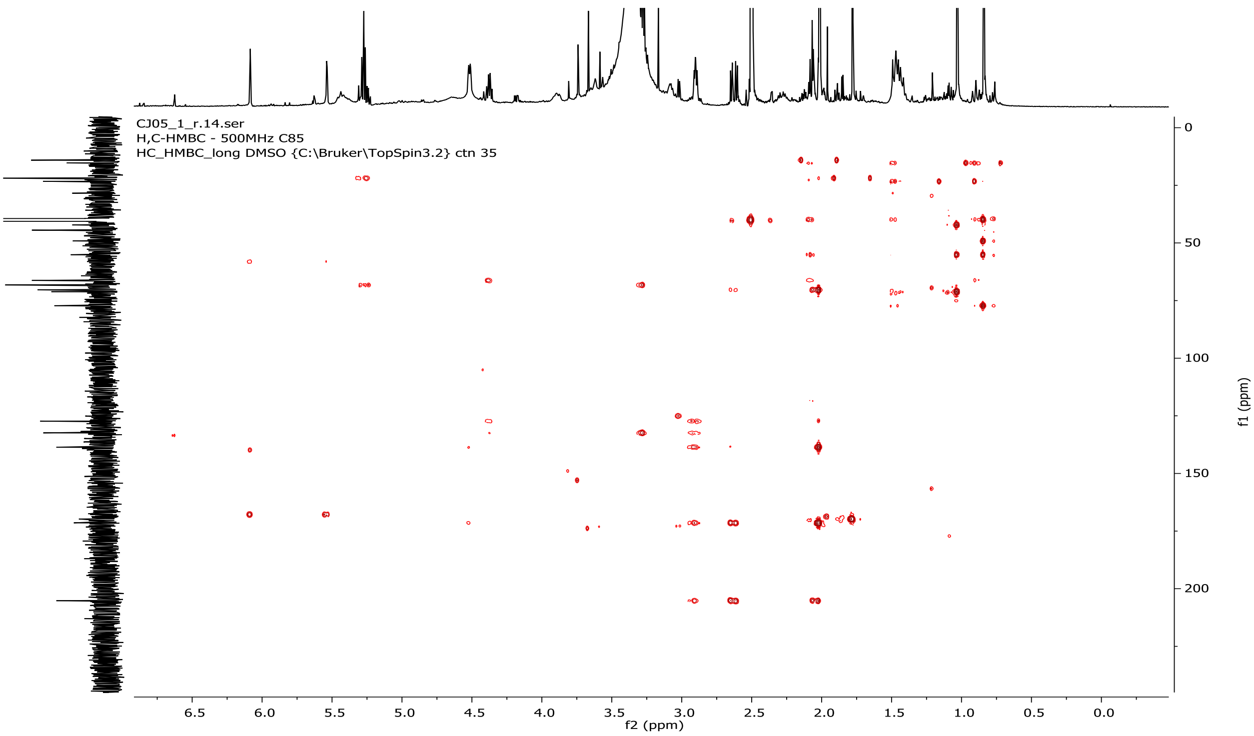
Appendix 14: DEPT spectrum for compound 3



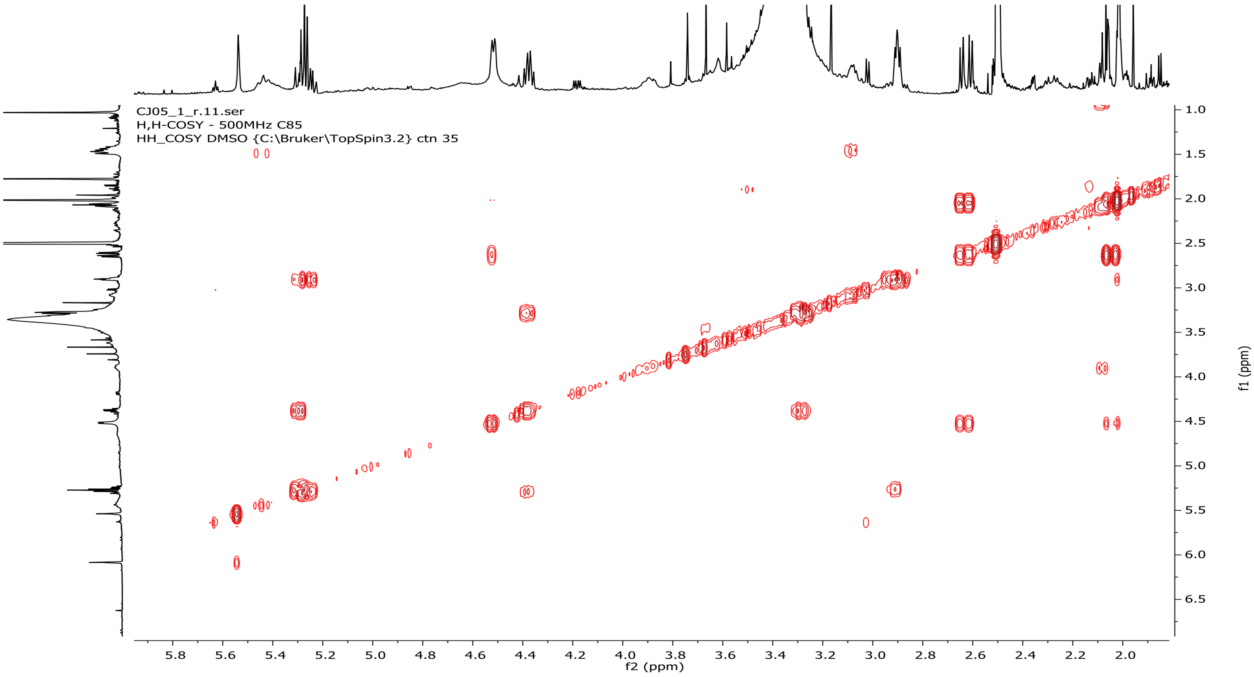
Appendix 15: HSQC spectrum for compound 3



Appendix 16: HMBC spectrum for compound 3



Appendix 17: ^{1}H-^{1}H COSY spectrum


CJ05_1_r.11.ser
H,H-COSY - 500MHz C85
HH_COSY DMSO {C:\Bruker\TopSpin3.2} ctn 35
f2 (ppm)
f1 (ppm)

Appendix 18: Screening of C. cinerariaefolium dichloromethane fractions and isolated compounds against selected bacteria.

Extracts	Zone of inhibition in mm			
	MRSA	*S. aureus*	*P.aeruginosa*	*S.sonnie*
Fraction 1	11.5 12.5 12	7 6.5 7.5	11 12 12	6 6 6
Fraction 2	7 8 8	7 7 8	11.5 11 12	6 6 6
Fraction 3	12 11 10	6.5 6.5 6.8	18 16 18	6 6 6
Fraction 4	13 12 12	11 9.5 9	22 22 24	6 6 6
Compound 1	6 6 6	6 6 6	6 6 6	6 6 6
Compound 2	6 6 6	6 6 6	6 6 6	6 6 6
Compound 3	6 6 6	6 6 6	8 8 7	6 6 6
Compound Mixtures	7 8 7	8 8.5 8	14 14 14	6 6 6
Positive control	26 28 26	24 23.5 25	25 26 27	23.5 24.5 26
Negative contol	6 6 6	6 6 6	6 6 6	6 6 6

Appendix 20: UV-vis absorbance values of silver nitrate, dichloromethane-Ag NPs, dichloromethane-methanol-Ag NPs, and dichloromethane-ethyl acetate-Ag NPs.

Silver Nitrate		Dichloromethane-Ag NPs		Dichloromethane-methanol-Ag NPs		Dichloromethane-ethyl acetate Ag NPs	
Wavelength (nm)	Abs	Wavelength (nm)	Abs	Wavelength (nm)	Abs	Wavelength (nm)	Abs
599.9831	0.04083	599.993	0.304338	599.9998	0.04985	599.993	0.41033
594.9855	0.04014	594.9949	0.322372	595.0016	0.04973	594.9949	0.42811
589.9814	0.03993	589.9901	0.341528	589.997	0.05024	589.9901	0.45018
585.008	0.03978	585.0161	0.362307	584.9858	0.05029	585.0161	0.47053
579.991	0.04014	579.9985	0.385357	580.0054	0.04997	579.9985	0.49269
575.0049	0.04011	575.0118	0.41014	574.9815	0.05106	575.0118	0.51841
570.0126	0.03988	569.9816	0.43906	569.9887	0.05117	569.9816	0.54293
565.0141	0.04082	564.9824	0.46772	564.9896	0.05316	564.9824	0.56722
560.0096	0.04031	560.0145	0.495469	559.9843	0.05269	560.0145	0.59381
554.9988	0.04047	555.0032	0.526765	555.0104	0.05304	555.0032	0.62126
549.9822	0.04056	549.986	0.559209	549.9932	0.05359	549.986	0.6495
544.9971	0.04057	545.0002	0.592993	545.0075	0.05479	545.0002	0.67738
540.0061	0.04084	540.0087	0.62741	540.016	0.05503	540.0087	0.70563
535.0094	0.04069	535.0114	0.662677	535.0187	0.05618	535.0114	0.7335
530.007	0.0409	530.0084	0.695801	530.0158	0.05692	530.0084	0.76093
524.999	0.04133	524.9997	0.729444	525.0071	0.05854	524.9997	0.7887
519.9853	0.04132	519.9855	0.766897	519.9929	0.05953	519.9855	0.81722
515.0039	0.04128	515.0034	0.799385	515.0109	0.06041	515.0034	0.84416
510.017	0.04233	510.016	0.831536	509.9857	0.06152	510.016	0.87041
504.987	0.04195	504.9853	0.862038	504.9929	0.06241	504.9853	0.89533
499.9895	0.04264	499.9872	0.890839	499.9948	0.06317	499.9872	0.92141
494.9867	0.042	494.9838	0.91957	494.9914	0.06508	494.9838	0.94484
490.0167	0.04286	490.0131	0.945681	489.9828	0.06645	490.0131	0.96837
485.0036	0.04248	484.9995	0.968052	485.0072	0.06812	484.9995	0.98985
479.9853	0.0426	480.0186	0.986816	479.9884	0.06921	480.0187	1.00824
475.0002	0.04348	474.9948	1.004065	475.0026	0.07017	474.9948	1.02789
470.0101	0.04309	470.0042	1.01923	468.9634	0.07507	470.0042	1.04218
465.0152	0.04331	465.0086	1.030178	463.9118	0.07924	465.0086	1.05489
460.0154	0.04404	460.0083	1.039016	458.8603	0.08193	460.0083	1.06509
455.0109	0.04439	455.0031	1.042721	453.7285	0.0847	455.0031	1.07243
450.0017	0.04484	449.9933	1.044212	448.677	0.08887	449.9933	1.07648
444.9878	0.04461	445.0171	1.041956	443.6254	0.09295	445.0171	1.0755
440.0076	0.04446	439.998	1.039147	438.4937	0.09573	439.9981	1.07373
434.9847	0.04469	435.0128	1.031613	432.1592	0.0985	435.0129	1.06978
429.9955	0.04517	429.9847	1.020906	428.3104	0.0998	429.9847	1.06154
425.002	0.0457	424.9906	1.007606	423.2589	0.10119	424.9906	1.05462
420.0042	0.04588	419.9922	0.994985	419.4903	0.0998	419.9923	1.04548

415.0021	0.04666	414.9895	0.97816	414.3585	0.0998	414.9895	1.03511
409.9958	0.04694	409.9826	0.961066	410.5899	0.0985	409.9826	1.02193
404.9853	0.04712	405.01	0.943689	405.4582	0.09711	405.01	1.01087
400.0092	0.04791	399.9948	0.925819	400.4066	0.09573	399.9948	1.0014
394.9905	0.04733	395.0142	0.9082	395.3551	0.09294	395.0142	0.98904
390.0065	0.04832	389.9909	0.891128	389.9993	0.09242	389.9909	0.98046
385.0186	0.04838	385.0024	0.872998	385.0108	0.09612	385.0024	0.97257
379.988	0.04948	380.0099	0.857212	380.0184	0.10084	380.01	0.96528
374.9923	0.04977	375.0137	0.841223	374.9834	0.1087	375.0137	0.96099
369.9929	0.04989	370.0137	0.826366	369.9834	0.11622	370.0137	0.96048
364.9897	0.05033	365.0099	0.816815	365.0185	0.12654	365.01	0.95985
359.9829	0.0515	360.0026	0.818198	360.0111	0.13947	360.0026	0.96738
355.0114	0.05275	354.9916	0.830159	355.0002	0.15427	354.9916	0.97121
349.9974	0.05367	350.0159	0.855266	349.9857	0.1709	350.0159	0.98595
345.0188	0.05405	344.9979	0.893345	345.0065	0.18677	344.9979	1.00499
339.9979	0.0553	340.0153	0.934162	339.9851	0.20444	340.0153	1.02712
335.0125	0.05685	334.9904	0.989802	334.9991	0.21837	334.9905	1.05022
329.9849	0.06165	330.0012	1.045508	330.0099	0.2314	330.0012	1.08881
324.993	0.067	325.0087	1.112012	325.0174	0.23963	325.0087	1.13576
319.9978	0.07533	320.0129	1.174931	319.9826	0.24501	320.0129	1.18318
314.9995	0.08425	315.014	1.24038	314.9838	0.24778	315.014	1.2523
309.9981	0.09309	310.012	1.308791	309.9818	0.25017	310.012	1.32972
304.9935	0.09887	305.007	1.390484	305.0158	0.25375	305.007	1.42871
299.986	0.10171	299.9988	1.48248	300.0077	0.25992	299.9988	1.53817

Appendix 21: UV-vis absorbance values of methanol-Ag NPs, ethyl acetate -Ag NPs, aqueous-Ag NPs, and Dichloromethane plant extract.

Methanol-Ag NPs		Ethyl acetate-Ag NPs		Aqueous-Ag NPs		Dichloromethane plant extract	
Wavelength (nm)	Abs	Wavelength (nm)	Abs	Wavelength (nm)	Abs	Wavelength (nm)	AbS
599.9998	0.22061	599.993	0.14704	599.9831	0.08689	599.9998	0.05777
595.0016	0.22874	594.9949	0.1522	594.9855	0.08907	595.0016	0.05711
589.997	0.23638	589.9901	0.15884	589.9814	0.09138	589.997	0.05747
584.9858	0.24596	585.0161	0.16588	585.008	0.09547	584.9858	0.05841
580.0054	0.25504	579.9985	0.17362	579.991	0.09799	580.0054	0.05918
574.9815	0.26432	575.0118	0.18102	575.0049	0.10264	574.9815	0.05978
569.9887	0.27541	569.9816	0.18931	570.0126	0.10831	569.9887	0.06041
564.9896	0.28484	564.9824	0.20417	565.0141	0.11323	564.9896	0.06101
559.9843	0.2966	560.0145	0.20736	560.0096	0.11869	559.9843	0.06151
555.0104	0.30735	555.0032	0.21704	554.9988	0.12579	555.0104	0.06224
549.9932	0.31848	549.986	0.22737	549.9822	0.1334	549.9932	0.06279
545.0075	0.33007	545.0002	0.23698	544.9971	0.14308	545.0075	0.06353
540.016	0.34099	540.0087	0.24692	540.0061	0.15331	540.016	0.06456

535.0187	0.35189	535.0114	0.25943	535.0094	0.16365	535.0187	0.06522
530.0158	0.36219	530.0084	0.2704	530.007	0.17579	530.0158	0.06558
525.0071	0.37197	524.9997	0.27851	524.999	0.18974	525.0071	0.06654
519.9929	0.38214	519.9855	0.29013	519.9853	0.20453	519.9929	0.06818
515.0109	0.39144	515.0034	0.30254	515.0039	0.21998	515.0109	0.06881
509.9857	0.41241	510.016	0.31307	510.017	0.23682	509.9857	0.07004
504.9929	0.41241	504.9853	0.32529	504.987	0.25498	504.9929	0.0708
499.9948	0.42629	499.9872	0.33697	499.9895	0.27356	499.9948	0.07164
494.9914	0.43975	494.9838	0.34783	494.9867	0.29289	494.9914	0.07262
489.9828	0.44669	490.0131	0.36044	490.0167	0.31423	489.9828	0.07305
485.0072	0.45363	484.9995	0.37385	485.0036	0.33497	485.0072	0.07523
479.9884	0.46752	480.0187	0.3816	479.9853	0.35671	479.9884	0.07566
475.0026	0.47402	474.9948	0.39348	475.0002	0.37806	475.0026	0.0769
470.012	0.48097	470.0042	0.40663	470.0101	0.39796	470.012	0.0782
465.0165	0.49485	465.0086	0.41823	465.0152	0.41547	465.0165	0.08034
460.0161	0.49485	460.0083	0.42906	460.0154	0.42954	460.0161	0.08157
455.0111	0.49485	455.0031	0.43886	455.0109	0.43998	455.0111	0.08314
450.0013	0.4943	449.9933	0.44781	450.0017	0.44686	450.0013	0.08502
444.9869	0.48736	445.0171	0.45275	444.9878	0.45121	444.9869	0.08594
440.0061	0.47255	439.9981	0.45208	440.0076	0.45025	440.0061	0.08876
434.9826	0.45774	435.0129	0.4586	434.9847	0.4475	434.9826	0.09042
429.9929	0.42117	429.9847	0.45718	429.9955	0.44127	429.9929	0.09212
424.9988	0.39158	424.9906	0.45265	425.002	0.43435	424.9988	0.09513
420.0004	0.3698	419.9923	0.44988	420.0042	0.42535	420.0004	0.09837
414.9977	0.37721	414.9895	0.4448	415.0021	0.41398	414.9977	0.10181
409.9909	0.39206	409.9826	0.43847	409.9958	0.40081	409.9909	0.10693
405.0184	0.39896	405.01	0.43083	404.9853	0.38804	405.0184	0.11333
400.0031	0.42117	399.9948	0.42729	400.0092	0.37541	400.0031	0.12226
394.9839	0.45033	395.0142	0.42069	394.9905	0.36509	394.9839	0.13361
389.9993	0.47995	389.9909	0.41533	390.0065	0.35897	389.9993	0.14843
385.0108	0.49431	385.0024	0.41424	385.0186	0.35668	385.0108	0.16904
380.0184	0.53133	380.01	0.41408	379.988	0.36104	380.0184	0.19566
374.9834	0.59958	375.0137	0.41963	374.9923	0.37373	374.9834	0.23074
369.9834	0.64056	370.0137	0.43579	369.9929	0.39445	369.9834	0.27534
365.0185	0.6913	365.01	0.46458	364.9897	0.42489	365.0185	0.33032
360.0111	0.75899	360.0026	0.5118	359.9829	0.4619	360.0111	0.4033
355.0002	0.83347	354.9916	0.58036	355.0114	0.49779	355.0002	0.49068
349.9857	0.91169	350.0159	0.66822	349.9974	0.53211	349.9857	0.5891
345.0065	0.9859	344.9979	0.76237	345.0188	0.55524	345.0065	0.69131
339.9851	1.05396	340.0153	0.85489	339.9979	0.56837	339.9851	0.7938
334.9991	1.12153	334.9905	0.93423	335.0125	0.58058	334.9991	0.87066
330.0099	1.18203	330.0012	0.99562	329.9849	0.58823	330.0099	0.93416
325.0174	1.22081	325.0087	1.03015	324.993	0.59116	325.0174	0.96679
319.9826	1.25859	320.0129	1.02806	319.9978	0.58851	319.9826	0.97166
314.9838	1.2861	315.014	1.02278	314.9995	0.58208	314.9838	0.96001
309.9818	1.32102	310.012	1.01873	309.9981	0.57446	309.9818	0.94463

305.0158	1.37794	305.007	1.03286	304.9935	0.57466	305.0158	0.94583
300.0077	1.44258	299.9988	1.05886	299.986	0.58849	300.0077	0.95908

Appendix 22: UV-vis absorbance values of dichloromethane-methanol plant extract, dichloromethane-ethyl acetate plant extract, methanol plant extract, and ethyl acetate plant extract

Dichloromethane-methanol plant extract		Dichloromethane-ethyl acetate plant extract		Methanol plant extract		Ethyl acetate plant extract	
Wavelength (nm)	Abs	Wavelength (nm)	Abs	Wavelength (nm)	Abs	Wavelength (nm)	Abs
599.9998	0.16314		0.29542	599.9998	0.05389	599.9998	0.08992
595.0016	0.1653	595.0016	0.29742	595.0016	0.05363	595.0016	0.09151
589.997	0.16725	589.997	0.30134	589.997	0.0549	589.997	0.09178
584.9858	0.16992	584.9858	0.30412	584.9858	0.05472	584.9858	0.09227
580.0054	0.17197	580.0054	0.30691	580.0054	0.05474	580.0054	0.09302
574.9815	0.17452	574.9815	0.30937	574.9815	0.05585	574.9815	0.09339
569.9887	0.1781	569.9887	0.31305	569.9887	0.05645	569.9887	0.09409
564.9896	0.18117	564.9896	0.31527	564.9896	0.05576	564.9896	0.09368
559.9843	0.18311	559.9843	0.32054	559.9843	0.05752	559.9843	0.09551
555.0104	0.18679	555.0104	0.32309	555.0104	0.05846	555.0104	0.09667
549.9932	0.19024	549.9932	0.327	549.9932	0.05998	549.9932	0.09722
545.0075	0.19298	545.0075	0.33106	545.0075	0.06074	545.0075	0.09911
540.016	0.19689	540.016	0.33488	540.016	0.06092	540.016	0.10029
535.0187	0.20031	535.0187	0.33863	535.0187	0.06289	535.0187	0.10073
530.0158	0.20392	530.0158	0.34311	530.0158	0.06378	530.0158	0.10222
525.0071	0.20693	525.0071	0.34681	525.0071	0.06433	525.0071	0.10392
519.9929	0.2112	519.9929	0.35205	519.9929	0.06628	519.9929	0.10522
515.0109	0.2157	515.0109	0.35616	515.0109	0.06728	515.0109	0.10682
509.9857	0.2207	509.9857	0.36074	509.9857	0.06836	509.9857	0.10853
504.9929	0.22417	504.9929	0.36537	504.9929	0.07016	504.9929	0.1097
499.9948	0.22999	499.9948	0.37119	499.9948	0.0717	499.9948	0.11186
494.9914	0.23436	494.9914	0.37624	494.9914	0.07285	494.9914	0.11385
489.9828	0.23918	489.9828	0.3814	489.9828	0.07449	489.9828	0.11558
485.0072	0.24436	485.0072	0.38706	485.0072	0.07647	485.0072	0.11798
479.9884	0.24978	479.9884	0.3929	479.9884	0.07922	479.9884	0.11981
475.0026	0.25498	475.0026	0.39872	475.0026	0.08135	475.0026	0.12183
470.012	0.26216	470.012	0.40499	470.012	0.08338	470.012	0.12457
465.0165	0.26875	465.0165	0.41315	465.0165	0.08614	465.0165	0.12741
460.0161	0.27582	460.0161	0.41954	460.0161	0.08902	460.0161	0.13027
455.0111	0.28332	455.0111	0.42685	455.0111	0.0921	455.0111	0.13348
450.0013	0.29223	450.0013	0.4357	450.0013	0.09447	450.0013	0.13672
444.9869	0.30058	444.9869	0.44244	444.9869	0.09804	444.9869	0.13972
440.0061	0.31058	440.0061	0.45177	440.0061	0.10156	440.0061	0.14351

434.9826	0.32008	434.9826	0.46189	434.9826	0.10549	434.9826	0.14765
429.9929	0.33141	429.9929	0.4714	429.9929	0.10884	429.9929	0.15197
424.9988	0.34502	424.9988	0.48241	424.9988	0.11308	424.9988	0.15648
420.0004	0.35678	420.0004	0.49486	420.0004	0.11826	420.0004	0.16322
414.9977	0.36864	414.9977	0.50986	414.9977	0.12432	414.9977	0.16998
409.9909	0.38327	409.9909	0.52791	409.9909	0.13162	409.9909	0.17957
405.0184	0.40086	405.0184	0.54892	405.0184	0.14145	405.0184	0.19329
400.0031	0.42104	400.0031	0.57464	400.0031	0.15298	400.0031	0.20917
394.9839	0.44698	394.9839	0.608	394.9839	0.17231	394.9839	0.23424
389.9993	0.47949	389.9993	0.64929	389.9993	0.19631	389.9993	0.26953
385.0108	0.51924	385.0108	0.69701	385.0108	0.23557	385.0108	0.31915
380.0184	0.57333	380.0184	0.75887	380.0184	0.28858	380.0184	0.39369
374.9834	0.63802	374.9834	0.83246	374.9834	0.36327	374.9834	0.50182
369.9834	0.71614	369.9834	0.91458	369.9834	0.46481	369.9834	0.66596
365.0185	0.80465	365.0185	1.00863	365.0185	0.58883	365.0185	0.88576
360.0111	0.92382	360.0111	1.12808	360.0111	0.75404	360.0111	1.21372
355.0002	1.05919	355.0002	1.2609	355.0002	0.94684	355.0002	1.64479
349.9857	1.20759	349.9857	1.41409	349.9857	1.15077	349.9857	2.133
345.0065	1.35126	345.0065	1.56242	345.0065	1.3475	345.0065	2.6343
339.9851	1.50556	339.9851	1.68934	339.9851	1.55003	339.9851	3.06244
334.9991	1.63032	334.9991	1.81309	334.9991	1.70783	334.9991	3.05468
330.0099	1.77195	330.0099	1.88032	330.0099	1.83004	330.0099	3.104
325.0174	1.80501	325.0174	1.93031	325.0174	1.85583	325.0174	3.18641
319.9826	1.83973	319.9826	1.9276	319.9826	1.82033	319.9826	3.1912
314.9838	1.81105	314.9838	1.9035	314.9838	1.75252	314.9838	3.195
309.9818	1.79496	309.9818	1.87221	309.9818	1.70172	309.9818	3.23028
305.0158	1.7825	305.0158	1.86844	305.0158	1.66407	305.0158	3.43286
300.0077	1.81374	300.0077	1.89122	300.0077	1.64976	300.0077	3.17316

Appendix 23: UV-vis absorbance values of aqueous plant extract, hexane plant extract, and dichloromethane-hexane plant extract

Aqueous plant extract		Hexane plant extract		Dichloromethane-hexane plant extract	
Wavelength (nm)	Abs	Wavelength (nm)	Abs	Wavelength (nm)	Abs
599.9831	0.05654	599.9998	0.07941	599.9998	0.09948
594.9855	0.05723	595.0016	0.07938	595.0016	0.09967
589.9814	0.05737	589.997	0.08019	589.997	0.10031
585.008	0.05716	584.9858	0.08113	584.9858	0.10095
579.991	0.05762	580.0054	0.08266	580.0054	0.10277
575.0049	0.05722	574.9815	0.08252	574.9815	0.10357
570.0126	0.05852	569.9887	0.08389	569.9887	0.10529
565.0141	0.06213	564.9896	0.08353	564.9896	0.10713
560.0096	0.05996	559.9843	0.08559	559.9843	0.10799
554.9988	0.06032	555.0104	0.08657	555.0104	0.10935
549.9822	0.06151	549.9932	0.08765	549.9932	0.11117

544.9971	0.06233	545.0075	0.08906	545.0075	0.11352
540.0061	0.06255	540.016	0.08965	540.016	0.11496
535.0094	0.06331	535.0187	0.09149	535.0187	0.11636
530.007	0.06427	530.0158	0.09245	530.0158	0.11807
524.999	0.06516	525.0071	0.09364	525.0071	0.11925
519.9853	0.06575	519.9929	0.09551	519.9929	0.12179
515.0039	0.06672	515.0109	0.0964	515.0109	0.12402
510.017	0.06814	509.9857	0.09767	509.9857	0.1261
504.987	0.06947	504.9929	0.09905	504.9929	0.12808
499.9895	0.07026	499.9948	0.10085	499.9948	0.12981
494.9867	0.07198	494.9914	0.1025	494.9914	0.13173
490.0167	0.07356	489.9828	0.10422	489.9828	0.1337
485.0036	0.07509	485.0072	0.10551	485.0072	0.13632
479.9853	0.07673	479.9884	0.10778	479.9884	0.13891
475.0002	0.07855	475.0026	0.10891	475.0026	0.14168
470.0101	0.08006	470.012	0.1108	470.012	0.14559
465.0152	0.08275	465.0165	0.11275	465.0165	0.14761
460.0154	0.08555	460.0161	0.11505	460.0161	0.15188
455.0109	0.08804	455.0111	0.11761	455.0111	0.15692
450.0017	0.09061	450.0013	0.11891	450.0013	0.1615
444.9878	0.09441	444.9869	0.121	444.9869	0.16788
440.0076	0.09811	440.0061	0.12357	440.0061	0.17504
434.9847	0.10296	434.9826	0.12606	434.9826	0.18088
429.9955	0.10839	429.9929	0.12814	429.9929	0.18993
425.002	0.11464	424.9988	0.13065	424.9988	0.19935
420.0042	0.12334	420.0004	0.13406	420.0004	0.20844
415.0021	0.13524	414.9977	0.13727	414.9977	0.21619
409.9958	0.14953	409.9909	0.13963	409.9909	0.22222
404.9853	0.17004	405.0184	0.14304	405.0184	0.22761
400.0092	0.19684	400.0031	0.14607	400.0031	0.2377
394.9905	0.23836	394.9839	0.15001	394.9839	0.24818
390.0065	0.28983	389.9993	0.15464	389.9993	0.26167
385.0186	0.36782	385.0108	0.15867	385.0108	0.27951
379.988	0.47734	380.0184	0.16377	380.0184	0.30031
374.9923	0.62072	374.9834	0.17039	374.9834	0.32304
369.9929	0.80888	369.9834	0.17606	369.9834	0.34622
364.9897	1.04125	365.0185	0.18315	365.0185	0.36892
359.9829	1.30286	360.0111	0.19157	360.0111	0.39571
355.0114	1.57434	355.0002	0.20073	355.0002	0.42184
349.9974	1.87832	349.9857	0.20894	349.9857	0.45025
345.0188	2.1343	345.0065	0.21906	345.0065	0.47562
339.9979	2.29915	339.9851	0.23156	339.9851	0.5038
335.0125	2.50595	334.9991	0.24182	334.9991	0.52846
329.9849	2.64012	330.0099	0.25354	330.0099	0.55035
324.993	2.54214	325.0174	0.26522	325.0174	0.57072
319.9978	2.59506	319.9826	0.2776	319.9826	0.59025

314.9995	2.43125	314.9838	0.28877	314.9838	0.61128
309.9981	2.35127	309.9818	0.2998	309.9818	0.63446
304.9935	2.22077	305.0158	0.3156	305.0158	0.66258
299.986	2.15912	300.0077	0.3335	300.0077	0.70253

Appendix 24: UV-vis absorbance values of mixture of silver nitrate, hexane extract, and mixture of silver nitrate and dichloromethane-hexane plant extract.

Mixture of Ag NO_3 and hexane extract		Mixture of Ag NO_3 and dichloromethane-hexane extract	
Wavelength (nm)	Abs	Wavelength (nm)	Abs
599.993	0.08779	599.993	0.11064
594.9949	0.08846	594.9949	0.11282
589.9901	0.08904	589.9901	0.11477
585.0161	0.08997	585.0161	0.11751
579.9985	0.0916	579.9985	0.11915
575.0118	0.09252	575.0118	0.12239
569.9816	0.09344	569.9816	0.12493
564.9824	0.09465	564.9824	0.12733
560.0145	0.09502	560.0145	0.12897
555.0032	0.09679	555.0032	0.13116
549.986	0.09823	549.986	0.13366
545.0002	0.09934	545.0002	0.13562
540.0087	0.10097	540.0087	0.13709
535.0114	0.10133	535.0114	0.13948
530.0084	0.10357	530.0084	0.14136
524.9997	0.10443	524.9997	0.14214
519.9855	0.1061	519.9855	0.14438
515.0034	0.10775	515.0034	0.14597
510.016	0.10871	510.016	0.14657
504.9853	0.10994	504.9853	0.14771
499.9872	0.11201	499.9872	0.14904
494.9838	0.11305	494.9838	0.15003
490.0131	0.11488	490.0131	0.15192
484.9995	0.11691	484.9995	0.15265
480.0187	0.11769	480.0187	0.15404
474.9948	0.12037	474.9948	0.15418
470.0042	0.12259	470.0042	0.15657
465.0086	0.12412	465.0086	0.15709
460.0083	0.12653	460.0083	0.15995
455.0031	0.12795	455.0031	0.1615
449.9933	0.13136	449.9933	0.16291
445.0171	0.1341	445.0171	0.16538
439.9981	0.13633	439.9981	0.16831
435.0129	0.13917	435.0129	0.17054
429.9847	0.14298	429.9847	0.17367
424.9906	0.14555	424.9906	0.17728
419.9923	0.14877	419.9923	0.18072

414.9895	0.15317	414.9895	0.18542
409.9826	0.15836	409.9826	0.19007
405.01	0.16232	405.01	0.19509
399.9948	0.16796	399.9948	0.20119
395.0142	0.17305	395.0142	0.2065
389.9909	0.17836	389.9909	0.21335
385.0024	0.1859	385.0024	0.22146
380.01	0.19355	380.01	0.22958
375.0137	0.20089	375.0137	0.23865
370.0137	0.20982	370.0137	0.24691
365.01	0.22016	365.01	0.25652
360.0026	0.2319	360.0026	0.26873
354.9916	0.24567	354.9916	0.28234
350.0159	0.26157	350.0159	0.29676
344.9979	0.27949	344.9979	0.31382
340.0153	0.29841	340.0153	0.33224
334.9905	0.32094	334.9905	0.35653
330.0012	0.34676	330.0012	0.38196
325.0087	0.37508	325.0087	0.4165
320.0129	0.40755	320.0129	0.45575
315.014	0.43711	315.014	0.49776
310.012	0.47229	310.012	0.54684
305.007	0.51033	305.007	0.59771
299.9988	0.55554	299.9988	0.65599

Appendix 25: Diameter of dichloromethane-methanol-Ag NPs as determined by imageJ

Area	Mean	Min	max	radius squared	radius	Diameter
2868.417	74.493	0	255	913.5085987	30.22430477	**60.44860954**
1455.528	62.308	0	227	463.543949	21.53007081	**43.06014162**
858.271	103.109	0	255	273.3347134	16.53283743	**33.06567485**
422.131	122.17	26	224	134.4366242	11.59468086	**23.18936172**
3191.259	46.322	0	255	1016.324522	31.87984508	**63.75969016**
99.974	134.375	44	237	31.8388535	5.6425928	**11.2851856**
128.68	136.247	42	214	40.98089172	6.401631958	**12.80326392**
348.882	129.519	31	254	111.1089172	10.54082147	**21.08164293**
696.851	88.465	0	206	221.9270701	14.89721686	**29.79443371**
818.982	120.692	1	255	260.822293	16.14999359	**32.29998718**
333.501	115.19	20	254	106.2105096	10.30584832	**20.61169664**
948.119	99.654	0	255	301.9487261	17.37667189	**34.75334379**
1223.219	95.941	0	240	389.5601911	19.73727922	**39.47455844**
1408.777	119.399	0	255	448.6550955	21.18148001	**42.36296003**
391.75	145.088	35	250	124.7611465	11.16965293	**22.33930585**
690.074	124.573	27	255	219.7687898	14.82460083	**29.64920166**
857.662	97.54	0	251	273.1407643	16.52697082	**33.05394163**
1344.057	102.058	0	255	428.0436306	20.68921532	**41.37843064**
463.247	120.316	14	224	147.5308917	12.14622953	**24.29245905**
1507.914	76.565	0	255	480.2273885	21.9140911	**43.82818219**
293.299	108.778	1	255	93.40732484	9.664746496	**19.32949299**
1567.305	75.174	0	250	499.1417197	22.3414798	**44.6829596**
955.124	135.092	1	255	304.1796178	17.44074591	**34.88149182**
845.784	122.018	1	255	269.3579618	16.4121285	**32.82425699**
1034.007	110.046	0	255	329.3015924	18.14666891	**36.29333781**
382.232	101.687	1	220	121.7299363	11.03312904	**22.06625807**
539.77	118.892	3	255	171.9012739	13.11111261	**26.22222522**
158.071	118.218	6	220	50.3410828	7.095145016	**14.19029003**
184.263	129.705	2	255	58.68248408	7.660449339	**15.32089868**
563.45	131.189	15	255	179.4426752	13.39562149	**26.79124298**
215.329	108.736	24	221	68.57611465	8.281069656	**16.56213931**
241.827	118.276	24	223	77.01496815	8.775817236	**17.55163447**
391.369	118.326	18	247	124.6398089	11.16422003	**22.32844006**
1935.831	83.247	0	255	616.5066879	24.82955271	**49.65910542**
264.365	108.346	14	224	84.19267516	9.175656661	**18.35131332**
622.536	114.934	9	253	198.2598726	14.08047842	**28.16095685**
382.232	122.975	24	224	121.7299363	11.03312904	**22.06625807**
2284.866	80.01	0	255	727.6643312	26.97525405	**53.95050811**
2284.866	80.01	0	255	727.6643312	26.97525405	**53.95050811**
516.851	115.199	0	242	164.6022293	12.82974003	**25.65948006**
308.451	91.923	0	238	98.23280255	9.911246266	**19.82249253**
164.162	133.695	27	249	52.28089172	7.230552657	**14.46110531**
775.429	99.261	0	255	246.9519108	15.71470365	**31.4294073**
327.334	121.271	32	255	104.2464968	10.21011738	**20.42023475**

259.796	128.236	13	252	82.73757962	9.096019988	**18.19203998**
743.449	108.203	0	255	236.7671975	15.38724139	**30.77448277**
948.119	92.422	0	255	301.9487261	17.37667189	**34.75334379**
168.73	128.329	10	251	53.73566879	7.330461704	**14.66092341**
665.937	113.369	1	255	212.0818471	14.56303015	**29.1260603**
309.136	139.724	44	241	98.45095541	9.922245482	**19.84449096**
539.77	90.225	0	242	171.9012739	13.11111261	**26.22222522**
513.805	141.39	41	253	163.6321656	12.79187889	**25.58375779**
582.638	111.569	0	255	185.5535032	13.62180249	**27.24360499**
241.827	127.699	15	254	77.01496815	8.775817236	**17.55163447**
417.258	135.921	29	255	132.8847134	11.5275632	**23.0551264**
194.923	152.068	60	255	62.07738854	7.878920518	**15.75784104**
207.182	139.017	40	244	65.98152866	8.122901493	**16.24580299**
236.649	117.384	19	231	75.36592357	8.681354938	**17.36270988**
198.121	157.515	71	255	63.09585987	7.943290242	**15.88658048**
194.314	126.444	36	231	61.88343949	7.866602792	**15.73320558**
797.51	69.779	0	255	253.9840764	15.93687788	**31.87375575**
1628.523	98.103	0	255	518.6378981	22.77362286	**45.54724572**
120	72.456	2	172	38.21656051	6.181954425	**12.36390885**
215.329	120.154	21	234	68.57611465	8.281069656	**16.56213931**
140.406	128.206	55	222	44.71528662	6.686948977	**13.37389795**
140.406	142.111	53	225	44.71528662	6.686948977	**13.37389795**
243.197	137.044	6	255	77.45127389	8.800640538	**17.60128108**
164.923	143.553	49	255	52.52324841	7.247292488	**14.49458498**
137.36	138.098	65	242	43.74522293	6.614017155	**13.22803431**
507.714	84.75	0	255	161.6923567	12.71583095	**25.4316619**
268.096	113.645	0	202	85.38089172	9.240178122	**18.48035624**

Appendix 26: Diameter of dichloromethane-Ag NPs as determined by imageJ

Area	**Mean**	**Min**	**max**	**radius squared**	**radius**	**Diameter**
142.278	121.62	28	255	45.31146497	6.731379128	**13.46275826**
6072.878	72.315	0	255	1934.03758	43.97769411	**87.95538823**
125.612	150.25	56	255	40.00382166	6.324857442	**12.64971488**
139.191	149.731	35	255	44.32834395	6.657953435	**13.31590687**
139.191	145.84	12	255	44.32834395	6.657953435	**13.31590687**
157.4	123.695	18	245	50.12738854	7.080069811	**14.16013962**
163.187	147.757	48	255	51.97038217	7.209048631	**14.41809726**
91.971	155.763	49	255	29.29012739	5.41203542	**10.82407084**
157.4	101.991	0	200	50.12738854	7.080069811	**14.16013962**
355.231	106.768	0	242	113.1308917	10.63630066	**21.27260132**
3400.158	81.501	0	255	1082.852866	32.9067298	**65.8134596**
1702.393	97.368	0	255	542.1633758	23.28440198	**46.56880397**
142.278	140.21	35	255	45.31146497	6.731379128	**13.46275826**
1532.957	87.269	0	255	488.2028662	22.09531322	**44.19062644**
148.913	150.433	17	255	47.42452229	6.886546471	**13.77309294**
2304.064	117.041	0	255	733.7783439	27.08834332	**54.17668664**
112.418	143.142	23	255	35.80191083	5.983469798	**11.9669396**

176.844	137.375	25	255	56.31974522	7.504648241	**15.00929648**
145.441	137.337	28	255	46.31878981	6.805790903	**13.61158181**
313.258	161.627	59	255	99.76369427	9.988177725	**19.97635545**
121.6	154.827	48	255	38.72611465	6.223030986	**12.44606197**
3040.606	110.321	0	255	968.3458599	31.11825605	**62.23651211**
398.13	146.07	37	255	126.7929936	11.2602395	**22.520479**
1023.643	95.892	0	255	326.0009554	18.05549654	**36.11099309**
76.231	148.231	50	255	24.27738854	4.927209001	**9.854418001**
165.733	152.153	0	255	52.78121019	7.265067804	**14.53013561**
262.025	155.602	60	255	83.44745223	9.134957703	**18.26991541**
157.4	163.09	55	255	50.12738854	7.080069811	**14.16013962**
123.914	170.048	65	255	39.46305732	6.281962856	**12.56392571**
1154.578	160.844	44	255	367.7	19.17550521	**38.35101042**
2841.232	74.278	0	255	904.8509554	30.08074061	**60.16148121**
91.971	158.463	62	255	29.29012739	5.41203542	**10.82407084**
121.6	162.891	48	255	38.72611465	6.223030986	**12.44606197**
91.971	163.636	57	255	29.29012739	5.41203542	**10.82407084**
3067.765	57.137	0	231	976.9952229	31.2569228	**62.5138456**
339.491	149.266	25	255	108.1181529	10.39798792	**20.79597585**
91.971	145.921	53	255	29.29012739	5.41203542	**10.82407084**
292.425	161.377	31	255	93.12898089	9.650335792	**19.30067158**
121.6	148.428	42	255	38.72611465	6.223030986	**12.44606197**
1007.98	111.203	0	247	321.0127389	17.91682837	**35.83365674**
174.684	159.53	55	255	55.63184713	7.458675964	**14.91735193**
130.704	151.217	57	255	41.62547771	6.451780972	**12.90356194**
121.6	161.481	48	255	38.72611465	6.223030986	**12.44606197**
1702.393	148.501	29	255	542.1633758	23.28440198	**46.56880397**
398.13	135.958	23	255	126.7929936	11.2602395	**22.520479**
91.971	157.799	58	255	29.29012739	5.41203542	**10.82407084**
262.025	156.63	53	255	83.44745223	9.134957703	**18.26991541**
157.4	169.465	59	255	50.12738854	7.080069811	**14.16013962**
119.285	168.962	62	255	37.9888535	6.163509836	**12.32701967**
109.717	165.677	60	255	34.94171975	5.91115215	**11.8223043**
121.6	176.73	73	255	38.72611465	6.223030986	**12.44606197**
91.971	146.437	33	247	29.29012739	5.41203542	**10.82407084**
107.711	157.181	43	255	34.30286624	5.856864882	**11.71372976**
197.522	155.363	48	255	62.90509554	7.931273261	**15.86254652**
960.76	121.382	11	255	305.9745223	17.49212744	**34.98425488**
2444.953	80.866	0	236	778.6474522	27.90425509	**55.80851018**
138.265	166.199	62	255	44.03343949	6.635769698	**13.2715394**
1379.877	144.544	18	255	439.4512739	20.96309314	**41.92618627**
163.804	147.981	61	255	52.16687898	7.222664258	**14.44532852**
121.6	143.028	26	245	38.72611465	6.223030986	**12.44606197**
119.285	163.647	72	255	37.9888535	6.163509836	**12.32701967**
265.112	129.46	18	255	84.43057325	9.188611062	**18.37722212**
121.6	158.08	59	255	38.72611465	6.223030986	**12.44606197**
102.156	166.06	68	255	32.53375796	5.703837126	**11.40767425**
148.913	166.769	53	255	47.42452229	6.886546471	**13.77309294**

Appendix 27: Diameter of dichloromethane- ethyl acetate-Ag NPs as determined by imageJ

Area	**Mean**	**Min**	**max**	**radius squared**	**radius**	**Diameter**
5093.056	100.102	0	255	1621.992357	40.27396624	**80.54793248**
869.136	155.22	70	255	276.7949045	16.63715434	**33.27430868**
297.531	139.692	53	234	94.75509554	9.734222904	**19.46844581**
920.679	79.796	0	255	293.2098726	17.12337212	**34.24674423**
387.654	144.904	70	223	123.4566879	11.11110651	**22.22221302**
252.16	159.511	75	255	80.30573248	8.961346578	**17.92269316**
1077.16	135.398	33	255	343.044586	18.52146285	**37.04292569**
848.302	143.012	52	255	270.1598726	16.43654077	**32.87308155**
3233.951	119.085	28	254	1029.920701	32.09237761	**64.18475522**
349.383	164.935	75	255	111.2684713	10.54838714	**21.09677429**
2968.519	121.42	16	255	945.3882166	30.74716599	**61.49433198**
426.852	166.301	91	255	135.9401274	11.65933649	**23.31867298**
5697.299	94.121	0	255	1814.426433	42.59608472	**85.19216943**
3112.346	77.037	0	255	991.1929936	31.48321765	**62.9664353**
7976.929	95.679	0	255	2540.423248	50.40261152	**100.805223**
2257.407	92.342	0	227	718.9194268	26.81267288	**53.62534575**
406.173	150.289	50	255	129.3544586	11.37341016	**22.74682031**
2592.901	122.444	7	255	825.7646497	28.73612099	**57.47224198**
3533.025	120.198	7	255	1125.167197	33.543512	**67.087024**
1241.358	112.323	15	255	395.3369427	19.88308182	**39.76616364**
2565.741	113.049	0	255	817.1149682	28.5852229	**57.1704458**
2904.938	127.039	0	255	925.1394904	30.41610577	**60.83221155**
327.392	155.402	0	255	104.2649682	10.2110219	**20.42204379**
900	143.184	38	255	286.6242038	16.92997944	**33.85995888**
313.272	88.756	11	161	99.76815287	9.988400916	**19.97680183**
332.099	158.303	70	255	105.7640127	10.2841632	**20.5683264**
344.753	175.502	89	255	109.793949	10.47826078	**20.95652157**
297.531	171.205	85	255	94.75509554	9.734222904	**19.46844581**
780.556	104.165	30	255	248.5847134	15.76656949	**31.53313897**
350.926	170.045	81	255	111.7598726	10.5716542	**21.14330841**
445.062	165.075	64	255	141.7394904	11.90543953	**23.81087906**
1358.333	138.008	0	255	432.5901274	20.7988011	**41.59760221**
1590.432	116.134	0	255	506.5070064	22.50571053	**45.01142106**
4177.238	94.141	0	255	1330.330573	36.47369701	**72.94739401**
1493.21	133.966	7	251	475.544586	21.8069848	**43.6139696**
2929.012	109.942	0	255	932.8063694	30.54187894	**61.08375789**
1714.429	88.553	0	203	545.9964968	23.36656793	**46.73313586**
1590.432	117.767	8	252	506.5070064	22.50571053	**45.01142106**
5394.522	106.278	0	255	1718.000637	41.44877124	**82.89754247**
176.852	173.458	84	255	56.32229299	7.504817985	**15.00963597**
558.025	150.681	69	255	177.7149682	13.33097776	**26.66195553**
3785.494	114.984	0	255	1205.571338	34.72133836	**69.44267672**
1087.963	58.706	0	198	346.4850318	18.61410841	**37.22821682**

883.333	135.608	44	255	281.316242	16.77248467	**33.54496934**
445.062	164.146	77	255	141.7394904	11.90543953	**23.81087906**
278.704	145.743	51	255	88.75923567	9.421212006	**18.84242401**
3020.679	51.3	0	255	961.9996815	31.0161197	**62.03223941**
2258.025	98.836	0	255	719.116242	26.81634282	**53.63268563**
330.556	141.04	55	243	105.2726115	10.26024422	**20.52048844**
1834.877	139.082	40	255	584.3557325	24.17345098	**48.34690197**
733.025	139.261	31	255	233.4474522	15.27898728	**30.55797456**
599.074	152.242	62	253	190.7878981	13.81259925	**27.6251985**
731.481	138.647	49	246	232.9557325	15.26288742	**30.52577485**
204.012	139.414	53	253	64.97197452	8.060519495	**16.12103899**
230.556	152.496	58	255	73.42547771	8.568866769	**17.13773354**
263.272	154.064	66	251	83.84458599	9.156668935	**18.31333787**
469.444	147.295	59	253	149.5044586	12.22720158	**24.45440317**
406.79	144.693	52	240	129.5509554	11.38204531	**22.76409062**
294.444	133.943	34	231	93.77197452	9.683593058	**19.36718612**
128.395	97.012	31	162	40.89012739	6.394538872	**12.78907774**
151.852	161.305	74	255	48.36050955	6.954172097	**13.90834419**
241.127	165.968	72	255	76.79203822	8.763106653	**17.52621331**
164.352	171.514	91	255	52.34140127	7.234735743	**14.46947149**
106.481	154.189	79	245	33.9111465	5.823327785	**11.64665557**
2402.778	116.847	0	255	765.2159236	27.66253646	**55.32507293**
2667.515	101.399	0	255	849.5270701	29.14664766	**58.29329533**
576.852	151.824	69	255	183.710828	13.55399675	**27.10799351**
575.463	152.515	67	255	183.2684713	13.53766861	**27.07533722**
731.481	122.809	22	255	232.9557325	15.26288742	**30.52577485**
174.691	181.202	102	255	55.63407643	7.458825406	**14.91765081**
701.235	143.883	47	255	223.3232484	14.94400376	**29.88800752**
297.531	137.165	59	233	94.75509554	9.734222904	**19.46844581**
760.185	130.276	15	255	242.0971338	15.55947087	**31.11894174**
582.407	168.655	59	255	185.4799363	13.61910189	**27.23820378**
263.272	145.162	67	255	83.84458599	9.156668935	**18.31333787**
258.333	174.15	97	255	82.27165605	9.070372432	**18.14074486**
128.395	87.02	21	163	40.89012739	6.394538872	**12.78907774**
1669.136	131.229	19	255	531.5719745	23.05584469	**46.11168939**
7221.296	110.809	5	255	2299.775796	47.95597769	**95.91195538**
4241.358	126.974	9	255	1350.750955	36.75256393	**73.50512786**
2345.988	91.777	0	255	747.1299363	27.33367769	**54.66735539**
232.716	174.986	77	255	74.1133758	8.608912579	**17.21782516**
232.716	174.986	77	255	74.1133758	8.608912579	**17.21782516**
535.802	139.138	62	222	170.6375796	13.06283199	**26.12566398**
114.506	152.865	66	255	36.46687898	6.03878125	**12.0775625**
313.272	169.37	61	255	99.76815287	9.988400916	**19.97680183**
313.272	169.37	61	255	99.76815287	9.988400916	**19.97680183**
349.383	160.599	77	255	111.2684713	10.54838714	**21.09677429**
17038.349	60.873	0	255	5426.225796	73.66292009	**147.3258402**
512.037	77.341	0	143	163.0691083	12.76985154	**25.53970307**
2411.728	107.438	0	255	768.066242	27.71400805	**55.42801609**
279.321	161.925	75	255	88.95573248	9.431634667	**18.86326933**
470.062	145.03	65	254	149.7012739	12.23524719	**24.47049439**

263.272	133.482	31	255	83.84458599	9.156668935	**18.31333787**
151.852	155.369	85	239	48.36050955	6.954172097	**13.90834419**
233.179	124.148	62	218	74.26082803	8.617472253	**17.23494451**
2763.889	123.705	16	255	880.2194268	29.66849216	**59.33698431**
87.346	180.799	93	255	27.81719745	5.27420112	**10.54840224**
708.025	119.887	42	213	225.4856688	15.01618023	**30.03236047**
171.528	164.686	81	255	54.62675159	7.390991246	**14.78198249**
249.074	153.111	66	252	79.32292994	8.906342119	**17.81268424**
232.716	168.094	58	255	74.1133758	8.608912579	**17.21782516**
114.969	128.224	51	210	36.61433121	6.050977707	**12.10195541**
397.917	167.939	78	255	126.7251592	11.25722698	**22.51445396**
1428.086	134.164	10	255	454.8044586	21.32614495	**42.65228991**
2695.062	134.965	0	255	858.3	29.2967575	**58.593515**
1084.877	127.69	24	255	345.5022293	18.58769026	**37.17538053**
247.84	165.083	76	255	78.92993631	8.884252152	**17.7685043**
749.074	134.077	17	255	238.5585987	15.4453423	**30.8906846**
1594.83	117.555	14	255	507.9076433	22.53680641	**45.07361283**
3446.605	112.211	7	255	1097.644904	33.13072448	**66.26144896**
1220.988	138.217	30	255	388.8496815	19.71927183	**39.43854366**
232.716	159.022	72	253	74.1133758	8.608912579	**17.21782516**
2906.173	125.017	35	255	925.5328025	30.42257061	**60.84514122**
3261.111	68.556	0	255	1038.570382	32.22685809	**64.45371617**
1056.481	80.286	0	170	336.4589172	18.3428165	**36.685633**
426.852	117.052	42	216	135.9401274	11.65933649	**23.31867298**
383.333	158.687	76	255	122.0805732	11.04900779	**22.09801559**
3033.642	121.816	13	246	966.1280255	31.08260004	**62.16520009**
557.099	149.501	68	255	177.4200637	13.3199123	**26.6398246**
379.321	47.717	0	135	120.8028662	10.99103572	**21.98207144**
375.926	179.009	90	255	119.7216561	10.94173917	**21.88347834**
185.494	140.262	64	242	59.07452229	7.685995205	**15.37199041**

Appendix 28: Diameter of aqueous-Ag NPs as determined by imageJ

Area	**Mean**	**Min**	**max**	**radius squared**	**radius**	**Diameter**
420.589	139.664	27	255	133.9455414	11.57348441	**23.14696882**
623.118	142.523	25	255	198.4452229	14.0870587	**28.17411741**
512.565	142.278	27	255	163.2372611	12.77643382	**25.55286764**
309.122	113.017	10	207	98.44649682	9.922020803	**19.84404161**
696.82	110.045	0	255	221.9171975	14.8968855	**29.79377099**
736.717	116.814	0	247	234.6232484	15.31741651	**30.63483301**
1188.98	92.111	0	241	378.656051	19.45908659	**38.91817318**
387.393	108.996	7	255	123.3735669	11.10736543	**22.21473087**
213.188	109.506	15	231	67.89426752	8.239797784	**16.47959557**
387.393	133.103	5	255	123.3735669	11.10736543	**22.21473087**
280.494	88.139	5	202	89.32929936	9.451417849	**18.9028357**
213.188	118.386	34	206	67.89426752	8.239797784	**16.47959557**
258.567	92.127	4	201	82.34617834	9.074479508	**18.14895902**
236.639	103.313	2	198	75.36273885	8.681171514	**17.36234303**

283.844	102.415	0	222	90.39617834	9.507690484	**19.01538097**
283.844	116.273	10	212	90.39617834	9.507690484	**19.01538097**
452.568	128.937	24	254	144.1299363	12.00541279	**24.01082558**
236.639	155.676	54	253	75.36273885	8.681171514	**17.36234303**
452.568	73.461	0	195	144.1299363	12.00541279	**24.01082558**
994.674	97.722	0	255	316.7751592	17.79817854	**35.59635707**
761.995	134.067	15	255	242.6735669	15.5779834	**31.1559668**
392.875	131.808	7	255	125.1194268	11.18567954	**22.37135908**
1409.02	69.18	0	229	448.7324841	21.18330673	**42.36661346**
362.115	171.841	74	255	115.3232484	10.73886625	**21.47773251**
620.377	131.269	27	255	197.572293	14.05604116	**28.11208231**
236.639	131.405	41	246	75.36273885	8.681171514	**17.36234303**
283.844	133.087	42	255	90.39617834	9.507690484	**19.01538097**
482.414	122.536	0	255	153.6350318	12.39495994	**24.78991987**
736.717	134.321	0	255	234.6232484	15.31741651	**30.63483301**
336.228	124.579	42	255	107.0789809	10.34789741	**20.69579483**
698.647	145.647	33	255	222.4990446	14.91640186	**29.83280373**
174.51	138.88	45	255	55.57643312	7.454960303	**14.90992061**
119.994	148.519	38	255	38.21464968	6.181799874	**12.36359975**
213.188	107.746	1	209	67.89426752	8.239797784	**16.47959557**
576.369	132.659	21	255	183.5570064	13.54832116	**27.09664233**
903.917	122.924	8	255	287.8716561	16.96678096	**33.93356191**
1557.186	100.316	0	255	495.9191083	22.2692413	**44.53848261**
623.118	41.355	0	238	198.4452229	14.0870587	**28.17411741**
2712.664	66.162	0	255	863.9057325	29.39227335	**58.78454669**
990.106	139.493	35	255	315.3203822	17.7572628	**35.5145256**
818.946	108.581	0	255	260.810828	16.14963863	**32.29927727**
362.115	139.63	28	255	115.3232484	10.73886625	**21.47773251**
308.513	112.964	0	255	98.25254777	9.912242318	**19.82448464**
362.115	101.926	10	236	115.3232484	10.73886625	**21.47773251**
194.915	128.88	27	255	62.07484076	7.878758834	**15.75751767**
1231.922	91.41	0	255	392.3318471	19.80736851	**39.61473701**
258.567	160.493	62	255	82.34617834	9.074479508	**18.14895902**
180.905	120.069	37	242	57.61305732	7.590326562	**15.18065312**
736.717	121.828	8	255	234.6232484	15.31741651	**30.63483301**
548.198	78.152	0	232	174.5853503	13.21307498	**26.42614995**
860.67	106.868	0	214	274.0987261	16.55592722	**33.11185444**
585.962	108.258	0	239	186.6121019	13.66060401	**27.32120802**
309.122	150.781	35	255	98.44649682	9.922020803	**19.84404161**
308.513	122.351	11	246	98.25254777	9.912242318	**19.82448464**
174.51	160.415	73	255	55.57643312	7.454960303	**14.90992061**
174.51	135.959	43	255	55.57643312	7.454960303	**14.90992061**
684.486	104.988	0	255	217.989172	14.76445637	**29.52891274**
236.639	112.157	0	213	75.36273885	8.681171514	**17.36234303**
331.964	150.711	0	255	105.7210191	10.2820727	**20.56414541**
119.994	125.043	31	215	38.21464968	6.181799874	**12.36359975**
309.122	21.848	0	188	98.44649682	9.922020803	**19.84404161**
215.32	103.451	1	191	68.57324841	8.280896594	**16.56179319**

Appendix 29: Diameter of methanol-Ag NPs as determined by imageJ

Area	Mean	Min	max	radius squared	radius	Diameter
4360.969	51.177	0	255	1388.843631	37.26719242	**74.53438483**
2708.363	43.291	0	191	862.5359873	29.36896299	**58.73792599**
620.283	111.689	37	213	197.5423567	14.05497623	**28.10995245**
410.259	78.718	9	176	130.6557325	11.43047385	**22.8609477**
724.406	78.8	2	170	230.7025478	15.18889554	**30.37779108**
750.214	68.156	0	168	238.9216561	15.4570908	**30.9141816**
1101.441	95.058	0	216	350.7773885	18.72905199	**37.45810398**
536.333	113.583	28	235	170.8066879	13.06930327	**26.13860654**
724.109	106.777	27	213	230.6079618	15.18578157	**30.37156313**
620.283	136.655	42	255	197.5423567	14.05497623	**28.10995245**
581.423	126.263	0	255	185.1665605	13.60759202	**27.21518403**
1439.615	69.102	0	195	458.4761146	21.41205536	**42.82411072**
445.856	123.853	29	255	141.9923567	11.91605458	**23.83210915**
981.893	120.858	19	255	312.7047771	17.68346055	**35.3669211**
604.265	151.44	48	255	192.4410828	13.87231353	**27.74462707**
784.624	66.374	0	145	249.8802548	15.80760117	**31.61520234**
469.885	141.394	51	252	149.6449045	12.23294341	**24.46588682**
582.906	118.302	49	234	185.6388535	13.62493499	**27.24986998**
559.768	94.127	9	255	178.2700637	13.35178129	**26.70356259**
253.038	134.911	61	240	80.58535032	8.97693435	**17.9538687**
335.801	154.594	19	255	106.9429936	10.34132456	**20.68264912**
559.768	119.826	32	228	178.2700637	13.35178129	**26.70356259**
497.176	99.291	26	177	158.3363057	12.5831755	**25.16635101**
390.977	98.687	41	175	124.5149682	11.15862752	**22.31725504**
652.618	77.241	18	180	207.8401274	14.41666145	**28.8333229**
1442.582	81.217	0	190	459.4210191	21.43410878	**42.86821756**
1061.69	62.062	0	191	338.1178344	18.3879807	**36.77596141**
468.921	106.555	12	193	149.3378981	12.22038862	**24.44077725**
1245.906	74.459	0	204	396.7853503	19.91947164	**39.83894328**
968.84	98.627	8	255	308.5477707	17.56552791	**35.13105582**

Appendix 30: Diameter of ethyl acetate-Ag NPs as determined by imageJ

Area	Mean	Min	max	radius squared	radius	**Diameter**
7507.06	132.784	46	255	2390.783439	48.89563825	**97.79127649**
1168.114	152.156	56	234	372.010828	19.28758222	**38.57516445**
3070.481	42.287	0	255	977.8601911	31.27075616	**62.54151233**
3228.5	56.172	0	185	1028.184713	32.06531948	**64.13063896**
3933.603	153.052	51	255	1252.739809	35.3940646	**70.7881292**
6148.939	58.188	0	255	1958.260828	44.25224094	**88.50448188**
5045.566	92.615	0	255	1606.868153	40.08575998	**80.17151995**
3369.796	129.902	13	255	1073.183439	32.75947862	**65.51895724**
4746.711	48.782	0	198	1511.691401	38.88047584	**77.76095167**
9063.241	61.717	0	255	2886.382484	53.72506384	**107.4501277**

Appendix 31: Bioassay results of Ag NPs against selected bacteria

		Test organism		
Ag NPs	***MRSA***	***S. aureus***	***P.aeruginosa***	***S.sonnie***
Dichloromethane-Ag NPs	7.4 7 7.5	9 9.5 10.5	13 14 13	9.5 10 9
Aqueous-Ag NPs	7 7.5 7.2	9.5 10 10	8 10 9	8 8.8 8
Dichloromethane-methanol-Ag NPs	7.6 7 8	10 11 11.5	9 9 10	9 10 9.5
Methanol-Ag NPs	7.5 7 7	8 8.5 8.5	9 10 9.5	8.5 8 8.2
Dichloromethane-Ethyl acetate-Ag NPs	6.8 6.5 7	8 8.5 8	8 7.5 8	8 7.5 8.5
Ethyl acetate-Ag NPs	6.5 6.8 6.5	7 7.5 7.2	7 7.5 7.5	7.8 7.5 7
Silver nitrate	6.5 6.5 6.2	7 7.2 7	7.5 6.5 7	7 6.8 7.5
Positive control	26 28 26	24 23.5 25	25 26 27	23.5 24.5 26
Negative contol	6 6	6 6	6 6	6 6

	6	6	6	6

Appendix 32: Wave numbers and % Transmittance of various Ag NPs.

Wave Numbers	% (Transmittance)					
	DCM-Ag NPs	Aqueous-Ag NPs	DCM-MEOH-Ag NPs	MEOH-Ag NPs	DCM-Ethly-Ag NPs	Ethly-Ag NPs
3999.64	9.08602	30.07722	14.01042	11.06987	26.8639	26.64641
3997.712	9.01193	29.93073	14.00506	10.63208	26.93794	25.81264
3995.783	9.2668	30.11729	13.6151	10.40091	26.88131	25.86228
3993.855	9.43592	30.22767	12.8736	10.38171	26.73606	25.64759
3991.927	8.9026	30.51852	13.22186	10.49184	26.09783	25.59293
3989.998	9.19674	29.88644	13.04065	10.69423	25.6789	25.00091
3988.07	9.40105	29.85026	13.26623	10.76839	26.37454	24.12817
3986.141	8.72307	30.18118	13.51812	10.78514	27.11769	24.36187
3984.213	8.293	30.50572	13.25706	10.82587	27.1922	25.60665
3982.284	8.14362	30.16053	13.42011	11.51299	26.32412	25.42056
3980.356	8.52595	29.43735	13.65263	11.08579	26.36034	24.92622
3978.427	9.31267	29.57388	13.43447	10.4048	26.56017	24.9734
3976.499	9.49394	29.17791	13.56629	10.41737	26.28318	24.88933
3974.57	9.25348	28.69447	12.96466	10.57928	26.78523	24.69565
3972.642	8.87891	29.41234	13.074	10.31583	26.22265	25.22593
3970.713	8.67749	30.14811	13.55789	10.57754	25.39316	25.12965
3968.785	8.49858	29.58361	13.53595	11.02346	25.67153	24.49783
3966.856	8.84661	29.53262	13.7381	11.06307	25.83604	25.13489
3964.928	9.12305	29.53425	14.14784	10.42387	26.46621	25.08207
3963	8.7829	29.5519	13.53567	9.82393	26.70064	25.29098
3961.071	9.00419	29.68383	12.58603	9.82543	26.45774	24.99521
3959.143	9.25232	29.97741	12.63719	10.25062	25.71943	24.93421
3957.214	9.04107	30.70305	13.73257	10.65635	25.32902	25.42086
3955.286	8.93171	29.94867	13.92512	9.89151	25.13531	25.00906
3953.357	9.56408	29.2415	13.87017	9.93231	25.65555	24.90362
3951.429	9.08204	29.44845	13.45352	10.35218	25.64044	24.99387
3949.5	8.96772	29.18678	13.15355	10.47179	25.56653	25.07506
3947.572	8.76639	29.27174	13.46174	9.81824	24.98287	25.01949
3945.643	8.69123	29.43642	14.16218	10.29189	25.27658	24.88957
3943.715	9.10889	29.28795	13.76299	11.39198	25.98455	24.69934
3941.786	9.18169	29.24416	14.2147	11.48669	25.62844	25.18745
3939.858	9.29751	30.10366	15.07069	11.92277	25.33992	25.52228
3937.929	8.83926	30.40368	14.71214	11.02005	26.96239	26.19499

3936.001	9.71731	29.93892	14.48205	10.30747	27.06747	26.47729
3934.073	9.86839	29.24798	13.77119	10.54752	26.65348	26.17113
3932.144	9.51208	28.72762	13.30689	10.29288	25.83109	24.42522
3930.216	9.54244	29.00064	14.15604	10.08698	25.38144	23.11653
3928.287	9.49346	29.71177	14.16795	10.66647	26.40986	24.59908
3926.359	10.0057	29.40033	13.27495	11.03888	27.55478	25.61832
3924.43	9.72787	28.72133	13.49083	10.87336	26.92245	24.621
3922.502	9.93482	28.66314	14.49863	10.74877	26.11561	24.79967
3920.573	10.05202	28.82087	14.62124	10.71081	26.19041	25.35893
3918.645	9.54209	28.9419	14.68077	10.79617	26.63612	24.9025
3916.716	9.81866	29.10018	14.76847	10.7234	26.49135	23.98361
3914.788	10.0104	30.10688	14.895	11.06863	25.94359	24.74829
3912.859	9.4385	30.56104	14.5775	12.07828	25.60568	26.13478
3910.931	9.63911	30.38318	14.61609	12.14439	27.54331	26.50049
3909.002	10.11623	30.32931	15.05902	11.76252	27.58604	26.78226
3907.074	10.15251	30.17413	14.4948	11.16255	26.85173	26.38609
3905.146	9.22612	27.79463	13.17692	10.29629	27.01054	24.44131
3903.217	8.9852	26.6477	12.66709	9.45657	25.76401	22.76884
3901.289	9.18699	27.17475	13.59323	10.70028	24.35319	22.65533
3899.36	9.08185	28.48809	14.10985	10.42478	24.34601	24.29126
3897.432	9.3713	28.64569	15.42576	11.63808	25.03703	24.86875
3895.503	10.45633	29.71042	15.88277	12.52315	26.51563	26.44413
3893.575	10.2095	29.15402	15.08761	12.56097	27.02379	27.20644
3891.646	9.02987	28.03988	13.89383	11.27404	27.11057	26.50947
3889.718	9.13178	29.94704	14.27957	10.62106	26.88957	25.62957
3887.789	10.97379	29.80936	14.63638	11.84954	25.78089	25.73161
3885.861	9.51828	28.06019	14.14834	11.64788	26.23883	24.61715
3883.932	8.22381	28.13416	14.25888	10.87406	26.03509	23.88508
3882.004	9.43919	28.32692	14.29491	11.29748	24.62977	25.44889
3880.075	9.15444	29.20831	14.78551	11.00058	25.58963	25.80713
3878.147	8.83968	30.35444	16.0404	10.53935	25.35535	25.91108
3876.219	10.23577	30.09098	15.23271	11.52626	26.09438	26.75327
3874.29	9.93768	29.25201	14.31598	11.69323	26.82947	26.6084
3872.362	9.53793	28.7061	14.51118	10.32485	25.49758	24.86081
3870.433	8.93347	27.41223	13.47213	11.55902	24.72958	23.50391
3868.505	8.037	28.00981	13.88426	10.66955	24.85208	23.94378
3866.576	9.5458	28.59451	14.77318	10.3439	24.99611	25.0901
3864.648	9.26841	28.20624	14.38391	9.9685	25.89608	24.19478
3862.719	8.4336	28.38258	14.08307	9.79742	24.84114	23.7121
3860.791	8.7403	29.70752	14.1049	10.6134	25.53735	25.98213
3858.862	9.74271	30.20213	15.26639	11.12369	26.53854	27.2858
3856.934	10.37699	28.35001	15.22353	10.71534	26.47899	26.79304
3855.005	10.0184	25.11204	13.62589	10.00465	24.90219	22.81021
3853.077	7.77576	21.73185	11.76002	9.43839	22.6137	16.12681
3851.148	6.40991	26.26837	14.26752	9.55255	20.08733	17.84709
3849.22	9.18201	29.68909	15.6454	11.19606	22.76421	25.23643

3847.292	10.37748	30.99791	15.75989	11.54218	25.67318	28.21366
3845.363	10.61079	30.64501	15.80055	11.42787	27.44857	27.75773
3843.435	9.84213	28.64164	14.71092	10.57767	27.30447	25.88059
3841.506	9.59534	27.05336	13.51896	10.18163	25.41861	24.57739
3839.578	8.66379	25.5495	12.59954	9.70498	23.52142	22.68036
3837.649	7.11741	25.05498	12.71198	9.26689	22.36105	21.21592
3835.721	6.13625	27.67832	13.98762	9.73767	21.67851	21.96421
3833.792	8.33688	30.16018	14.01915	10.1475	23.57983	24.93374
3831.864	9.31614	29.53716	14.36657	10.38917	25.63908	26.50104
3829.935	9.41253	29.76643	14.76233	9.98279	26.12367	26.39877
3828.007	9.94928	29.82499	14.74153	10.52338	25.93578	26.28465
3826.078	8.6582	28.50101	14.02709	10.7523	25.85252	25.43306
3824.15	8.23583	27.39193	14.8223	10.76546	24.97611	24.72896
3822.221	8.60949	26.06561	13.63382	10.95094	25.07484	23.66621
3820.293	6.70267	25.19557	12.0222	8.84473	23.34987	20.40018
3818.365	7.84086	27.1628	14.01207	9.41219	22.09266	22.74163
3816.436	7.45344	27.67493	12.33249	10.41775	22.74136	23.85965
3814.508	6.16394	29.92189	12.94126	8.58626	23.7694	23.35304
3812.579	8.2756	30.72143	15.19123	9.36881	24.81351	25.54839
3810.651	9.15601	30.38177	15.35879	10.22091	26.77104	27.20508
3808.722	9.57199	28.63515	14.60097	10.43253	26.58332	26.17062
3806.794	8.70485	26.90037	12.99412	9.5544	26.26861	24.27718
3804.865	8.26489	28.29961	14.09228	9.11851	23.85423	23.82542
3802.937	8.68433	28.02968	13.20124	9.82335	23.2971	23.46698
3801.008	6.97097	26.74479	11.61874	9.69045	23.13215	19.73416
3799.08	7.46103	28.15071	14.07458	9.61277	22.53314	21.48145
3797.151	8.32721	28.39244	14.48631	9.77577	23.36296	24.30299
3795.223	7.74397	30.36372	14.87198	9.6049	24.84086	25.86937
3793.294	9.45431	31.31318	15.54379	10.6711	25.40765	27.13412
3791.366	9.93287	31.87112	15.49033	10.84998	26.79572	26.87532
3789.438	9.0916	30.89256	15.1384	10.22319	26.37687	25.9749
3787.509	8.9689	30.02427	15.06628	9.737	25.71751	25.98629
3785.581	8.57997	29.62303	15.12061	9.78552	24.94345	25.41247
3783.652	8.1696	29.31639	14.82773	9.70454	24.24795	25.18948
3781.724	8.57876	29.75123	15.29013	9.86215	24.5984	25.70428
3779.795	7.7621	29.64222	14.33946	9.64529	24.88439	25.2007
3777.867	7.23209	29.93888	13.72977	9.08345	23.85263	24.27589
3775.938	9.08016	30.60088	15.22302	10.05411	24.78546	26.1748
3774.01	9.78926	30.60976	15.98301	10.83017	25.99096	27.29545
3772.081	9.68241	29.92727	15.71918	10.92686	26.23232	26.35808
3770.153	8.42909	28.28418	14.84169	10.28503	25.57345	24.02215
3768.224	8.4237	28.60372	15.40685	9.84949	25.12303	23.61623
3766.296	9.23329	29.29024	14.86559	10.36481	25.12959	24.82105
3764.367	9.44592	29.81962	14.71853	9.78374	25.98999	26.05348
3762.439	10.36168	29.91873	16.17215	10.30349	25.49494	26.56624
3760.511	10.1308	29.09245	15.57935	10.16598	26.4485	25.89164

3758.582	8.3411	28.7088	14.73546	9.58932	26.32062	24.32604
3756.654	8.13529	29.2606	15.56214	9.22987	25.49423	25.36309
3754.725	9.39292	29.02714	15.70005	9.63832	25.14321	25.93521
3752.797	10.22308	26.04102	14.46601	10.3031	24.96732	24.43293
3750.868	8.22308	23.1672	14.0856	10.5333	23.03728	20.57223
3748.94	8.20022	24.58484	14.62558	11.24744	22.05392	20.24939
3747.011	9.97023	26.57207	15.70405	11.35721	23.60677	23.99947
3745.083	9.19115	25.72639	14.45623	11.15661	24.19888	23.48034
3743.154	6.51108	27.76825	15.31304	10.00655	24.17206	22.66221
3741.226	10.24711	29.77489	16.63474	11.65808	24.08676	26.05742
3739.297	11.10281	29.52758	16.17288	11.55304	25.72539	26.74296
3737.369	9.85109	27.56938	14.82956	11.02014	26.13033	24.55214
3735.44	6.99174	24.07325	13.06096	10.2254	24.18673	19.5966
3733.512	7.11153	24.49281	13.52227	9.91419	21.68428	19.43638
3731.584	8.87727	29.78568	15.29564	10.38348	22.7834	23.97782
3729.655	10.29732	32.42551	17.70411	10.95879	25.57123	29.269
3727.727	10.52375	30.86823	17.55463	11.5702	26.56278	28.71793
3725.798	9.44251	29.86802	17.07979	10.63065	26.2267	26.04448
3723.87	8.73781	29.5388	16.09102	10.37317	24.59322	24.89017
3721.941	8.32896	29.61272	15.68855	9.65103	24.71452	25.23738
3720.013	8.81016	30.52514	16.8225	9.66404	24.73782	25.75284
3718.084	9.3959	31.18951	16.72358	10.28714	25.71123	26.28481
3716.156	9.86623	30.80342	15.68464	10.55682	26.08975	26.9081
3714.227	9.90705	28.7376	15.10619	10.6561	25.74566	26.16055
3712.299	8.421	25.90385	14.54567	10.49222	24.5561	23.2351
3710.37	5.43174	25.70993	14.6185	9.6179	22.18776	20.70768
3708.442	6.24475	29.64995	17.14726	9.1016	23.46805	23.60786
3706.513	9.09178	31.6302	17.56591	10.37742	26.13941	27.14555
3704.585	10.11996	31.08642	16.89426	11.13906	26.64505	27.35266
3702.656	9.4143	29.88555	15.89048	10.84799	25.86892	25.41166
3700.728	8.44905	29.99924	15.68895	9.91864	24.69485	24.08873
3698.8	9.40331	31.05589	16.24117	10.11722	24.5925	25.69968
3696.871	9.09296	30.99853	15.40339	10.29152	25.21441	26.6554
3694.943	9.18401	30.74882	15.04837	10.22679	25.66115	26.4141
3693.014	9.46598	28.90573	14.68175	10.2896	24.99204	25.17087
3691.086	8.22351	26.63105	13.82744	10.37261	24.05172	22.96182
3689.157	5.0174	24.54164	13.40378	9.30673	21.36694	19.18853
3687.229	4.96118	26.78902	14.89134	6.90358	20.69556	20.50677
3685.3	8.69563	30.892	16.57871	8.73406	23.50876	26.39851
3683.372	9.53898	32.24393	16.56671	10.25411	25.67078	28.08757
3681.443	9.6014	30.46566	15.2164	10.53523	25.52043	26.88227
3679.515	9.31053	28.35506	14.4339	10.25205	24.94129	25.81754
3677.586	8.51843	26.32048	13.42964	9.92551	23.92416	23.6066
3675.658	5.96519	23.53871	11.31157	8.81555	22.41298	19.33234
3673.729	4.81318	25.42889	13.30721	6.96231	19.74044	18.28773
3671.801	8.26383	27.37763	14.88137	10.44805	21.15252	22.91943

3669.873	5.81014	26.45544	13.46145	10.27312	20.93994	21.45214
3667.944	4.49147	29.41411	14.99206	7.53887	20.1817	21.95494
3666.016	7.69244	30.99769	16.62198	8.94354	23.35854	25.56987
3664.087	9.52605	30.69943	16.24492	9.52685	25.63932	27.23731
3662.159	9.38779	30.05898	15.91686	9.63376	25.01423	26.9709
3660.23	8.70509	29.76392	15.81252	9.28271	24.64805	25.60577
3658.302	8.10428	29.04341	14.87902	9.49906	24.4415	23.90911
3656.373	7.10629	27.25696	13.85641	8.73678	24.20473	22.35884
3654.445	7.65715	27.62853	14.63458	7.68819	23.47762	23.25079
3652.516	8.96052	27.23911	14.9286	9.48602	23.64215	24.51969
3650.588	6.54624	24.14954	12.61726	9.52864	22.84327	20.03298
3648.659	2.70313	22.42827	10.79265	7.91255	18.0028	15.48797
3646.731	2.52822	25.4867	12.00154	7.16963	16.31427	17.02927
3644.802	7.29483	29.88389	15.51174	7.66493	21.33968	23.02299
3642.874	9.02034	30.05306	15.80139	8.85743	24.35352	25.77878
3640.946	8.28074	29.43583	15.35378	9.21161	24.71293	25.44637
3639.017	8.20024	29.04174	15.2704	9.4782	24.68604	24.74358
3637.089	8.62047	28.8014	15.39367	9.53466	24.96561	24.72209
3635.16	8.62206	27.88192	14.39844	9.34079	23.94897	23.75519
3633.232	8.56705	27.37372	14.36956	8.76775	24.05433	23.25623
3631.303	8.88405	26.11002	14.41909	9.24238	23.03986	22.77761
3629.375	6.44262	23.97567	12.20757	8.93683	21.95319	19.35397
3627.446	3.59491	25.59886	12.40546	6.40518	20.23722	17.61336
3625.518	7.05968	29.07736	14.77186	8.2801	21.97048	23.19094
3623.589	8.74487	29.46286	15.64692	9.77594	24.44825	25.4711
3621.661	8.94572	27.42348	14.69741	9.57901	24.5606	24.65739
3619.732	6.9859	24.98345	13.71244	8.45245	22.63007	21.16326
3617.804	6.19343	25.30924	13.91265	7.23402	21.62923	19.54997
3615.875	7.94053	26.30947	13.95045	8.4634	22.499	21.30509
3613.947	8.32465	25.5735	13.73732	9.32587	22.87083	21.85145
3612.019	7.88576	25.39442	14.74754	8.41898	21.96764	21.59777
3610.09	8.27293	26.43133	15.62693	9.04239	21.91989	22.44084
3608.162	7.30938	26.7133	15.14806	8.53767	22.60874	22.07807
3606.233	7.40685	27.85211	15.9611	8.2717	23.80024	22.41451
3604.305	8.78822	28.17213	16.33667	9.67344	24.36631	23.87688
3602.376	9.19231	27.63444	15.12105	9.74063	24.22094	23.43042
3600.448	7.76479	26.28085	14.17991	8.39484	23.71458	21.63312
3598.519	7.97323	25.82597	14.88728	8.04173	23.17909	21.44972
3596.591	8.49548	25.0674	15.21561	8.71858	22.67587	21.89259
3594.662	8.37659	25.82852	15.09825	8.84925	23.5561	21.85849
3592.734	8.33821	27.03073	14.45463	8.49392	23.72283	21.90505
3590.805	8.91274	26.57058	14.18271	9.31533	23.96722	22.39231
3588.877	8.24414	24.45059	13.6139	9.74087	22.76007	20.71367
3586.948	5.70575	23.83654	13.8129	7.90752	20.7926	17.79586
3585.02	5.78463	25.19147	14.85186	7.5237	21.19225	19.86674
3583.092	8.21316	26.74672	15.02449	8.86544	23.20838	23.01909

3581.163	7.63959	26.04151	14.6329	8.86613	23.63019	22.88149
3579.235	7.26502	25.78169	14.464	8.28098	23.41619	22.27435
3577.306	7.71412	25.68009	14.05397	7.99302	23.31222	21.72043
3575.378	7.59121	25.12154	14.02997	8.56291	23.12315	21.29354
3573.449	7.1698	24.6516	13.98999	8.6542	23.45771	21.43107
3571.521	7.98662	24.96526	13.84322	8.61081	23.90223	21.81769
3569.592	8.2894	24.14638	13.30595	8.77223	23.44067	20.32869
3567.664	7.45313	21.97342	11.95911	8.86626	22.52158	17.98224
3565.735	6.04128	21.93777	12.56145	7.95842	20.27118	16.55781
3563.807	6.95324	24.39596	14.54508	8.24369	21.2772	19.3706
3561.878	7.42564	24.72534	14.53547	8.83649	22.72591	20.59547
3559.95	7.11833	24.19358	13.82654	8.7945	22.88618	20.14497
3558.021	7.40525	23.85944	13.36961	8.38103	22.29889	20.49767
3556.093	8.17877	24.15424	13.12669	8.30846	23.16952	20.34957
3554.165	7.73902	23.73536	12.87078	8.59683	23.40209	19.61239
3552.236	6.86214	22.9551	13.12875	8.58857	23.00181	19.2248
3550.308	7.43498	23.01467	13.10237	8.6682	22.33475	19.4254
3548.379	7.68991	22.53599	13.03828	8.48082	22.7177	19.69847
3546.451	5.99269	21.8462	12.95576	8.20662	21.79861	18.13441
3544.522	5.10907	22.16782	12.36531	7.47621	20.782	17.72837
3542.594	5.92766	23.07551	12.472	6.91599	20.90049	18.53455
3540.665	7.12515	23.42697	12.23386	7.40218	21.81108	19.92314
3538.737	7.37585	22.86094	12.55946	7.54102	22.77973	19.71166
3536.808	6.39792	22.32757	12.87096	7.78048	22.43739	18.40544
3534.88	5.73144	22.51874	12.14834	7.52439	22.04211	17.93788
3532.951	6.99693	22.92312	12.42625	6.85876	22.71828	18.88829
3531.023	6.82315	22.1739	12.83339	7.35093	22.14514	18.22912
3529.094	6.16262	21.87553	12.93043	7.93684	22.3268	17.92198
3527.166	5.4786	21.67512	12.98525	7.26209	22.04473	16.81667
3525.238	5.72444	21.35958	13.02865	7.41636	21.49263	17.16006
3523.309	5.69044	21.01369	12.3934	6.97424	20.42512	17.15423
3521.381	5.78437	21.96945	12.3208	7.2558	20.54921	17.70593
3519.452	6.05567	22.19161	12.22892	7.57771	21.59154	18.10081
3517.524	6.60825	22.30964	12.22637	7.31835	22.10875	18.18036
3515.595	6.06164	21.62916	11.96702	7.29235	21.45109	17.71286
3513.667	5.6288	20.77589	11.53044	6.91834	21.37132	17.25595
3511.738	5.43529	20.85799	11.8168	6.29665	21.03902	16.85954
3509.81	5.41951	21.12236	11.98077	6.22737	20.57834	16.98479
3507.881	5.51361	20.5265	12.04007	6.66706	20.70197	16.9379
3505.953	5.99382	20.2025	12.29042	7.12571	21.29813	17.49972
3504.024	5.84841	19.88919	11.75605	7.08216	21.08422	16.77068
3502.096	5.68076	19.90807	11.26531	6.49206	19.6984	15.52962
3500.167	5.09566	20.38007	11.36061	6.89643	20.18735	15.7621
3498.239	4.9708	20.3678	11.17882	7.30461	20.94943	15.60925
3496.311	5.45058	19.76859	11.09948	6.60779	20.6734	15.51601
3494.382	5.74649	19.77725	11.37348	6.69012	20.30471	15.60129

3492.454	5.6803	19.10219	11.15582	6.60322	20.50838	15.98457
3490.525	5.75249	19.43415	11.43486	7.39425	21.3185	16.87934
3488.597	5.07986	19.65199	10.6822	6.99635	20.76979	16.61287
3486.668	4.41618	19.86465	9.68671	6.37645	20.33922	15.8819
3484.74	4.9257	20.31256	10.02335	6.20217	20.06857	16.0974
3482.811	5.55594	20.24832	10.83648	6.58752	21.00461	15.64252
3480.883	4.50651	18.82276	10.53404	6.87246	20.44507	15.09139
3478.954	4.63413	18.90492	10.59243	7.09558	20.25928	15.4737
3477.026	5.58657	19.24454	10.97728	7.05082	21.15739	16.45895
3475.097	5.51709	19.45196	10.76131	6.92984	20.95951	16.81629
3473.169	5.38473	19.48845	10.88682	7.19663	20.67034	16.29517
3471.24	5.31253	19.71788	11.82302	7.20439	21.69143	16.06069
3469.312	4.76475	19.00948	10.87397	7.14272	20.56441	15.75854
3467.384	4.63078	19.40781	10.37088	6.86005	19.74116	15.38598
3465.455	5.0631	20.12869	10.37576	7.22217	19.90582	14.91966
3463.527	6.19527	20.10259	10.8517	7.22363	20.88049	15.44347
3461.598	5.47742	19.86819	11.2609	6.61478	20.89446	15.45201
3459.67	5.68278	19.7169	11.50382	6.40076	20.94843	16.29488
3457.741	6.08909	19.64573	11.62261	6.63956	20.99498	17.01719
3455.813	6.71378	19.54241	11.68173	6.39945	21.0397	16.7285
3453.884	6.2901	19.13548	11.50878	6.60759	21.0721	16.12637
3451.956	5.5444	19.82273	11.38601	6.89615	21.17496	16.64133
3450.027	5.48763	19.38006	11.57652	7.56729	21.44426	16.47191
3448.099	5.62375	19.35807	12.02558	7.676	21.11667	15.26242
3446.17	5.33252	19.81564	11.98057	7.18564	20.44838	14.73889
3444.242	6.19459	20.14924	12.07601	8.35855	21.01405	15.66753
3442.313	6.67823	20.49037	12.4244	8.58527	21.62873	16.12246
3440.385	6.05904	20.65463	12.79568	8.58134	21.43092	16.1249
3438.457	5.59393	20.64584	12.53807	7.54073	20.79886	16.47765
3436.528	5.75955	20.22365	11.81517	6.94469	20.86873	16.81475
3434.6	6.01445	19.16783	11.27023	6.89584	20.70803	16.10189
3432.671	6.30987	19.22597	11.54802	7.33357	21.13707	15.51375
3430.743	6.17765	20.03998	11.58934	7.83483	21.40415	15.76874
3428.814	6.28333	20.72894	11.98902	7.6847	21.59353	16.73931
3426.886	6.49323	20.50725	11.91062	7.5855	21.64669	16.89381
3424.957	6.54087	20.26321	11.8746	7.93851	21.96835	16.72491
3423.029	6.61248	20.22474	12.67833	8.25553	21.96722	15.98162
3421.1	6.55589	20.12201	12.7636	7.69288	21.64541	15.53934
3419.172	6.30243	20.12728	12.17299	7.29978	21.36992	16.41649
3417.243	6.38521	20.11694	11.63205	8.11261	21.80635	17.3196
3415.315	5.97343	20.64623	11.19048	8.7327	21.6838	16.47378
3413.386	6.17277	21.05913	11.04173	8.41283	21.45084	16.40355
3411.458	6.78785	20.46659	10.83416	7.75062	20.83441	15.98839
3409.53	6.79524	20.02346	10.91144	7.54161	21.63591	16.09192
3407.601	6.26238	20.38415	11.11384	7.16872	21.77777	16.33725
3405.673	6.96956	21.10167	12.09841	7.79465	21.82488	16.16819

3403.744	6.56482	21.04592	12.57294	8.13296	21.99164	16.28803
3401.816	6.22917	20.58288	12.30254	7.54836	21.64034	16.20928
3399.887	5.78061	20.59248	11.85366	7.02749	21.02229	15.91462
3397.959	5.92472	21.68954	11.27074	6.60131	21.60826	16.59108
3396.03	6.22331	21.3289	11.45508	6.40817	21.60758	16.82266
3394.102	6.48419	20.59294	12.22653	7.33944	21.6231	16.56759
3392.173	5.20075	20.87784	11.89193	7.22765	21.48324	16.30852
3390.245	6.02557	20.82976	11.80365	6.61679	21.1626	16.3568
3388.316	6.38626	20.89429	11.93002	7.0785	21.55336	16.80921
3386.388	6.26131	21.2446	12.19223	7.86284	21.76509	17.60461
3384.459	6.59144	21.35382	13.07415	7.34682	21.74854	17.41212
3382.531	7.39363	21.88965	13.23817	7.5454	22.23453	16.98384
3380.603	6.2132	21.87551	12.44445	6.81461	21.84677	16.99439
3378.674	6.359	22.22691	12.44338	6.45776	21.21582	17.11509
3376.746	6.45095	21.9107	12.79226	6.90669	21.04521	16.44852
3374.817	6.28013	21.63979	12.98057	6.39181	21.69262	16.72999
3372.889	6.04365	21.84406	12.59861	6.51492	21.50406	16.62647
3370.96	5.94556	21.92078	12.34588	6.65652	21.61215	16.34455
3369.032	6.06279	21.79387	12.20508	5.71046	20.60508	16.35104
3367.103	6.09171	21.97496	12.18405	5.4528	20.58378	16.60989
3365.175	5.86772	22.16581	12.29528	5.3902	21.35019	16.52792
3363.246	6.20021	22.28253	13.30741	6.54055	22.44352	17.54716
3361.318	5.34623	22.24122	13.00788	6.73757	22.16274	16.98334
3359.389	5.78945	22.74868	12.1524	6.06991	21.65339	16.26098
3357.461	6.3944	22.91721	12.25276	5.73501	21.26084	16.87527
3355.532	6.11123	22.86914	12.98929	6.42109	21.62346	17.31769
3353.604	5.48872	22.63019	13.1248	6.88736	21.51855	16.57853
3351.676	5.69106	22.85173	12.77698	6.58661	21.08175	16.36154
3349.747	5.47206	23.65367	12.98167	5.76902	21.12644	16.41154
3347.819	6.19841	23.7417	13.34913	5.92779	22.0602	16.63921
3345.89	6.09057	22.84076	13.37471	5.62635	21.86808	17.22495
3343.962	5.60593	22.46697	13.24274	5.19399	22.44309	17.41453
3342.033	5.45818	22.69307	12.85754	5.29493	22.69868	17.6984
3340.105	6.08511	22.28467	13.19965	6.10984	22.13299	17.71409
3338.176	5.88559	23.04385	13.45486	5.94125	21.74842	17.34973
3336.248	6.58524	23.51664	14.01015	5.835	22.32921	16.98384
3334.319	6.31758	23.72484	13.77607	5.72145	22.6512	17.25943
3332.391	6.19488	23.89149	13.93094	5.41582	22.50439	18.37817
3330.462	6.19097	23.63191	13.99959	5.50904	22.13052	17.9914
3328.534	6.6796	23.75646	13.73671	6.14243	22.03323	18.30396
3326.605	7.06395	24.33085	14.20073	6.426	22.16566	18.48087
3324.677	7.40828	24.46783	14.5415	5.90675	22.45833	18.68785
3322.749	7.59285	24.79349	14.0102	6.04006	22.90315	18.73725
3320.82	7.81943	25.19018	14.20859	6.46127	23.62444	18.26724
3318.892	7.40661	24.69778	14.65997	6.47916	23.09219	18.09706
3316.963	7.42518	24.03698	14.16471	6.762	22.74741	18.14275

3315.035	7.35169	23.75391	13.68486	6.39876	22.73293	18.40663
3313.106	7.43571	24.26397	14.05587	5.95143	22.5862	18.92943
3311.178	7.18044	24.1412	13.8071	6.13412	21.71304	18.67714
3309.249	6.55438	24.89138	14.05707	6.67449	22.17715	18.51475
3307.321	5.934	25.38396	14.1137	6.08649	22.84172	18.22982
3305.392	7.03651	25.39214	14.9078	5.57208	22.73649	18.86321
3303.464	7.586	25.36198	15.03452	5.60098	23.10807	19.26224
3301.535	7.84001	25.33799	15.05926	6.47477	23.52438	19.79159
3299.607	7.94517	25.07344	15.42036	6.8432	23.60274	19.38718
3297.678	8.28024	25.0796	15.77529	7.37081	24.1629	19.26608
3295.75	7.95005	25.48025	15.19586	7.19044	23.69263	19.08674
3293.822	7.84413	25.62619	15.44344	6.6954	23.57468	18.83764
3291.893	7.66383	25.54015	15.11504	6.27258	23.33614	19.09824
3289.965	8.27109	25.67913	15.15549	6.69782	23.98261	19.67741
3288.036	8.50788	25.24572	15.881	6.73349	23.97063	19.54952
3286.108	9.11247	25.74926	16.13295	7.52458	23.83562	19.87201
3284.179	9.06498	25.94514	15.72123	7.45441	24.01726	19.41427
3282.251	8.87241	25.85109	15.83493	7.5948	24.25172	19.8112
3280.322	8.08209	25.62727	15.26767	7.004	24.03476	20.21045
3278.394	8.52477	26.16638	15.66768	7.4735	24.68848	19.92481
3276.465	8.1256	25.63133	15.4179	7.98019	24.8466	19.9861
3274.537	8.68208	25.99694	15.72908	7.69867	24.62837	20.17343
3272.608	8.8675	26.13574	16.14417	7.54325	23.96744	19.3845
3270.68	9.07644	26.08796	15.8007	7.53576	24.45738	19.72167
3268.751	9.43557	25.86037	15.45023	7.13105	24.66369	20.22124
3266.823	9.73835	26.38629	15.61554	8.0585	24.81384	20.46066
3264.895	9.14646	26.54043	15.56495	8.32912	24.32829	19.56539
3262.966	8.99732	26.15992	15.70181	8.17736	24.54605	19.88241
3261.038	9.02813	25.35655	15.8501	7.43715	24.30492	20.50583
3259.109	9.65512	25.97636	16.34536	7.63937	24.72468	21.24119
3257.181	9.23712	25.98757	16.61158	7.8967	24.77643	20.73067
3255.252	9.32828	25.56779	17.18575	8.30084	24.80677	20.58916
3253.324	9.04034	25.52776	16.72554	8.32362	24.64536	20.25616
3251.395	9.30952	26.22974	16.27015	8.57493	24.64902	19.79937
3249.467	9.17247	26.24363	15.77079	8.23936	24.2877	19.10688
3247.538	9.04529	26.25768	15.31105	8.26532	24.52868	19.35617
3245.61	8.27961	25.4898	15.06158	7.93047	24.02098	20.12802
3243.681	8.79377	25.11227	14.78568	8.48877	23.94765	20.35862
3241.753	9.04297	25.91459	14.82068	8.49015	24.06184	19.95444
3239.824	9.31276	26.64743	15.25646	7.82277	24.37389	20.30366
3237.896	9.54995	26.35234	15.09187	7.6757	24.35551	19.83061
3235.968	8.96316	26.06719	14.74964	8.07935	24.31135	19.72302
3234.039	7.98917	25.60684	14.28202	8.0224	23.56492	19.74512
3232.111	8.69823	25.26533	15.2852	7.94487	23.90211	20.46132
3230.182	9.19584	25.57439	15.29439	7.16445	24.0539	19.84636
3228.254	9.35433	26.2328	15.26907	7.25305	24.14082	19.65094

3226.325	8.76502	25.84413	15.53974	8.35297	23.52792	19.82972
3224.397	8.82423	25.49145	15.0205	8.659	24.07904	20.72797
3222.468	8.90436	25.9638	14.40159	7.96	23.57796	19.8273
3220.54	9.16457	25.88124	14.90176	8.29867	23.53527	19.53841
3218.611	8.8323	25.37897	15.07752	7.80357	24.35662	19.90156
3216.683	9.17279	25.60458	15.86984	6.7151	25.179	19.98595
3214.754	8.8568	25.65097	15.92201	6.93815	24.19006	19.16338
3212.826	8.57058	25.48089	16.25445	7.3729	23.9476	19.17573
3210.897	8.28611	25.10876	15.73581	6.88532	24.25335	19.18295
3208.969	8.40557	24.96268	15.27697	6.5887	24.35356	18.53853
3207.041	8.90793	25.39524	14.90563	5.9585	23.27134	18.02782
3205.112	9.22898	25.89847	13.78306	6.68947	23.57337	19.43984
3203.184	9.34714	25.44238	14.05584	7.96774	24.10054	19.8808
3201.255	9.52694	25.85075	15.14315	8.20497	24.05262	19.73063
3199.327	8.85683	25.59287	15.16674	7.5907	23.50254	19.80362
3197.398	8.55928	25.5713	15.24653	7.53919	23.83788	19.69545
3195.47	8.37904	25.51184	15.25728	7.38804	23.55016	19.30375
3193.541	8.56444	25.41795	14.6223	7.18982	24.46392	19.50255
3191.613	9.10259	25.64845	14.23637	6.72967	24.7464	19.60268
3189.684	9.29728	26.49753	15.02456	7.44952	24.23424	19.41191
3187.756	8.62645	26.18117	15.09958	7.16239	23.98719	18.96752
3185.827	9.35843	25.64946	15.23734	7.71949	24.65762	19.1244
3183.899	10.07028	25.53239	15.07684	6.87548	24.73426	19.0287
3181.97	10.19157	25.33417	14.43977	6.80531	24.24953	19.9645
3180.042	9.61584	25.18579	14.84601	7.22228	23.99613	19.84382
3178.114	9.3646	24.83456	15.39362	7.37562	24.14021	19.25336
3176.185	9.19291	24.76132	14.87276	6.97291	24.39772	18.99247
3174.257	10.52612	25.43446	14.41352	7.03383	25.01426	19.86626
3172.328	10.15018	26.07395	14.49705	7.06128	24.89541	19.32383
3170.4	9.45592	25.99653	14.55126	6.68152	24.88797	19.66275
3168.471	9.43385	25.86907	14.86269	6.3246	24.52512	20.32096
3166.543	10.17029	26.69144	15.40364	7.26939	24.55172	20.55992
3164.614	9.92595	26.83062	15.13469	7.35736	24.62742	20.65748
3162.686	9.51256	26.44845	14.797	7.45438	25.41255	20.5308
3160.757	9.72119	26.42439	14.45139	7.29917	25.45964	20.47163
3158.829	10.23036	26.04511	14.7018	7.33759	25.86475	20.50275
3156.9	10.32651	25.67358	15.12749	7.66159	25.51183	20.62988
3154.972	10.39077	25.90997	15.65018	7.34784	25.26197	20.67406
3153.043	9.80382	26.12014	15.06863	7.04991	24.85802	20.90839
3151.115	10.77692	26.48997	15.41722	7.36709	26.04333	21.7648
3149.187	10.85333	26.29074	16.06612	7.36375	27.0824	21.51073
3147.258	11.4626	26.70359	17.079	8.05857	26.93676	21.54768
3145.33	10.90849	26.88067	16.85878	8.65264	26.10993	20.98203
3143.401	10.48656	27.02636	16.48115	8.19395	26.7138	21.41731
3141.473	10.48534	27.11895	16.44824	7.47447	26.47686	21.54181
3139.544	10.84229	27.69916	16.46288	7.85526	26.26164	21.86571

3137.616	11.13037	27.44644	16.29783	7.54031	26.74227	21.71105
3135.687	11.36857	26.79538	16.32039	8.1885	27.31142	21.39535
3133.759	11.39008	26.89175	16.07945	8.54727	26.3088	21.33173
3131.83	12.49335	27.43752	16.53464	8.75943	26.97057	21.82072
3129.902	12.33174	27.55863	16.37002	7.97589	27.19469	22.28545
3127.973	12.14622	28.07943	16.46623	8.82786	26.70378	23.05354
3126.045	11.94588	28.18368	17.07366	9.73405	27.11374	22.7183
3124.116	11.87309	28.00901	17.6476	8.95523	28.03187	22.61122
3122.188	11.15007	27.78852	16.71496	8.12352	27.72788	22.09604
3120.26	11.72929	28.38416	16.4026	7.82763	28.09586	22.5244
3118.331	12.24455	28.47049	16.00085	7.41475	28.22608	22.63885
3116.403	12.42048	28.78589	16.86185	7.90074	29.09972	23.14038
3114.474	12.47488	28.98619	16.58551	8.56359	28.69706	23.30771
3112.546	12.44676	29.43279	16.73796	9.11697	29.75123	23.92011
3110.617	12.54574	29.76866	17.30147	9.2256	29.25376	23.92109
3108.689	13.16412	29.68412	17.71885	9.05495	29.04933	24.70457
3106.76	13.58685	29.42654	17.60536	8.73377	29.82772	24.73069
3104.832	13.7159	29.23255	18.11357	9.02347	29.7681	24.11178
3102.903	13.66205	29.55103	18.11788	9.36656	28.9009	24.02453
3100.975	13.50259	30.81804	18.35693	9.09314	29.81121	24.81265
3099.046	13.44524	30.82224	19.08521	8.89315	30.99489	25.74127
3097.118	14.52987	30.80492	19.33723	9.38985	31.23125	26.35459
3095.189	13.66679	30.41629	18.86333	9.23355	30.59311	25.81262
3093.261	13.82062	30.77424	19.3259	9.0377	31.37374	26.06573
3091.333	13.91418	31.10803	18.596	8.67885	31.2798	26.07252
3089.404	14.44734	31.62988	18.34944	8.80581	31.40082	26.70739
3087.476	14.51385	31.82867	18.73546	9.20841	31.04084	27.23825
3085.547	14.52684	32.3502	19.36376	9.29867	31.75806	26.89723
3083.619	14.50386	32.24997	19.02193	8.56175	32.59329	26.86704
3081.69	15.30629	32.74493	19.14464	9.14262	32.55764	28.11121
3079.762	15.53101	33.53751	19.5934	9.3979	33.07472	28.61404
3077.833	16.02957	33.57957	20.44364	9.35697	34.40138	29.09022
3075.905	16.19539	32.95782	20.67192	9.10689	34.81369	28.87297
3073.976	16.80644	33.82837	20.36692	9.94722	35.40918	29.14383
3072.048	16.40393	33.95707	20.67133	10.37042	34.84378	29.71529
3070.119	16.79903	34.22531	21.51436	11.37213	34.20723	29.82058
3068.191	16.8789	34.17991	21.15331	10.93071	34.19365	28.98422
3066.262	17.15024	34.48712	21.39927	10.94854	35.89096	29.50245
3064.334	18.21561	35.27443	21.2849	10.76275	37.14087	30.27435
3062.406	18.23511	35.55318	20.78583	11.51199	37.2348	30.49229
3060.477	16.90889	35.44553	20.75436	11.68374	35.85202	30.19209
3058.549	16.91897	35.54131	21.05566	10.99356	35.4006	30.18738
3056.62	16.73437	35.6245	20.83647	10.06954	35.80221	29.86979
3054.692	17.57132	35.99232	20.67969	9.79455	36.73437	30.86577
3052.763	18.01962	36.75973	21.49113	10.21798	36.75311	31.3808
3050.835	18.17081	37.27797	22.18519	10.56488	36.61138	31.4687

3048.906	17.84618	36.48378	21.55392	10.81021	36.45488	31.65164
3046.978	17.3205	36.53393	21.51113	10.72962	36.7286	31.51145
3045.049	18.38103	37.17566	21.84981	11.22765	37.86527	31.38273
3043.121	19.06822	37.29226	22.29881	12.14344	38.82957	31.68682
3041.192	18.7957	36.75993	21.92954	12.27849	38.75882	31.50989
3039.264	19.53566	36.99997	21.92277	12.61965	39.13771	32.39725
3037.335	19.29627	36.4901	21.98546	12.13392	38.54842	32.46909
3035.407	19.09284	36.80053	21.57761	11.39901	38.22844	32.34952
3033.479	19.98869	37.42411	21.31992	11.84917	38.31313	31.95879
3031.55	20.22943	37.26465	22.37176	13.48136	38.69317	32.66252
3029.622	20.24463	37.00784	22.55458	13.55538	38.94909	33.01966
3027.693	20.40747	37.13197	22.51325	12.19076	39.02572	33.20688
3025.765	20.15732	37.02217	22.21276	11.66451	38.70979	32.48584
3023.836	19.60762	36.99312	22.65432	12.20288	38.96444	32.58244
3021.908	19.39008	37.57191	22.71563	11.69894	38.97322	33.07312
3019.979	19.86735	37.72511	22.71539	11.55382	39.63832	33.77684
3018.051	20.46656	37.71329	22.94787	12.55841	39.72841	32.93899
3016.122	20.84744	37.89621	23.12176	12.93913	40.25992	32.6972
3014.194	20.88974	38.26518	22.83814	13.05463	41.20143	33.93839
3012.265	21.95637	38.17615	23.4483	13.51	41.89618	35.04509
3010.337	22.34741	37.33087	23.49234	13.74167	42.03018	35.0064
3008.408	22.33791	37.78325	24.47791	14.47665	42.3324	35.28013
3006.48	21.73156	38.07774	24.45535	14.71609	42.26936	34.66746
3004.552	21.81022	38.82987	23.97559	14.6218	42.44581	33.98352
3002.623	21.71491	38.91702	23.22237	14.42604	41.94672	34.35757
3000.695	21.8344	38.53143	23.58573	14.0216	41.86992	35.5211
2998.766	22.1443	38.61423	23.78529	13.76626	41.70214	34.9938
2996.838	22.69205	39.42253	24.03664	14.35951	42.43965	35.8429
2994.909	22.64259	39.41928	24.25751	14.3989	42.8523	36.33313
2992.981	23.49815	39.84027	24.71971	14.61457	43.35824	36.88758
2991.052	24.20896	40.31208	25.46859	15.1651	43.85241	36.48576
2989.124	24.19115	40.17766	25.81193	15.35428	43.9573	35.82262
2987.195	24.11468	40.35284	25.74226	15.02427	43.69009	36.05064
2985.267	23.54844	41.07613	25.77412	15.2028	43.27204	36.634
2983.338	23.58579	41.03268	25.4221	14.72908	43.02122	36.92612
2981.41	24.54173	40.59364	25.55521	15.14991	43.84679	36.93469
2979.481	25.29477	40.49992	25.4796	16.18801	44.99274	36.89562
2977.553	25.27333	40.93629	25.50719	16.60673	45.71459	37.34019
2975.625	24.98833	42.204	25.30835	16.98528	45.89206	38.079
2973.696	25.41181	42.24823	25.98221	17.62262	46.26208	38.43035
2971.768	25.78458	41.8499	26.87975	17.32207	46.36728	38.24294
2969.839	25.36753	42.066	27.20391	17.09404	46.25403	38.39848
2967.911	25.37276	42.33675	27.13191	16.65488	46.22194	38.10791
2965.982	25.89929	42.9054	27.13015	16.73654	46.735	38.42036
2964.054	26.15675	42.92649	26.79098	16.94904	46.20023	39.31123
2962.125	26.39654	42.51673	26.72899	17.51415	46.48682	39.83281

2960.197	26.15669	41.83943	26.15648	17.51293	47.12122	39.53109
2958.268	26.41297	42.00354	26.17823	17.71659	47.55573	38.73544
2956.34	26.43553	41.96851	26.9098	17.68198	47.50898	38.77819
2954.411	26.614	42.42036	26.96855	17.34938	47.6567	39.30474
2952.483	27.10957	42.54411	26.9988	17.27947	47.91228	39.35636
2950.554	27.52774	42.89164	27.50215	18.00763	48.04629	40.17451
2948.626	27.0572	43.67756	27.63764	18.13625	48.15619	40.69654
2946.698	27.35059	43.97403	27.74691	18.30634	48.97043	41.19267
2944.769	28.28785	43.81016	27.34266	18.66733	49.2207	40.527
2942.841	28.6743	43.334	27.89175	19.1666	49.37054	40.43144
2940.912	28.06433	42.61901	27.93681	18.94533	48.81954	40.57576
2938.984	27.58021	42.65975	27.57857	18.6173	48.30726	40.2672
2937.055	27.9229	42.89975	26.97953	18.9572	48.71321	40.29029
2935.127	28.56569	43.44531	26.79243	19.58732	49.13044	40.47081
2933.198	28.22862	43.84638	27.35021	19.54754	49.17365	40.26106
2931.27	27.98345	43.46631	26.88017	19.29824	49.56686	39.56839
2929.341	28.56025	42.44484	25.86449	19.60411	50.5626	39.91651
2927.413	28.93609	42.86855	26.63106	19.8512	50.47404	40.45996
2925.484	28.87632	42.79177	27.03167	19.82117	49.15641	40.21177
2923.556	28.98743	42.70586	26.24825	19.60318	49.17684	40.49673
2921.627	29.25028	43.0323	25.74111	19.55135	49.24127	40.49381
2919.699	28.81799	43.74716	25.32485	19.30674	49.53525	40.20203
2917.771	28.15641	44.1366	25.20269	19.17777	50.41575	40.96692
2915.842	29.58367	44.23142	25.82938	20.06425	51.50648	41.85452
2913.914	30.98867	43.67169	26.13937	21.03979	52.14182	41.76519
2911.985	30.86876	43.4839	26.71095	21.84386	52.91811	41.87765
2910.057	30.5277	43.81592	27.30805	21.60489	52.47418	41.5698
2908.128	30.79985	44.22071	27.75821	21.29433	51.85415	42.0505
2906.2	30.90854	43.98053	28.13515	21.46249	51.90417	42.02042
2904.271	31.33106	43.95177	27.96789	21.53211	52.74804	42.25691
2902.343	31.45988	44.14891	28.28097	21.6817	52.9988	42.53566
2900.414	31.31001	44.24899	28.68863	21.62033	53.45701	42.81739
2898.486	31.68089	44.79107	28.66369	21.72536	54.19806	42.51245
2896.557	31.69634	44.95608	28.379	22.0625	53.81861	42.77131
2894.629	31.53817	44.65614	28.54428	22.2683	54.15868	43.40299
2892.7	31.65844	44.59778	28.89884	22.45735	54.11189	42.83532
2890.772	32.16583	44.38212	28.56072	22.64564	53.40179	42.26984
2888.844	31.9041	44.59934	28.86148	23.28006	53.49284	43.04168
2886.915	31.85979	44.93244	28.93875	23.29186	53.94014	43.39443
2884.987	32.1004	44.82204	28.86491	22.64182	54.6868	43.39735
2883.058	32.11744	44.58927	29.44337	23.07033	54.88818	43.21627
2881.13	32.17046	44.5795	29.35051	23.2741	54.38953	42.83352
2879.201	32.42785	44.48832	29.95435	23.0412	54.83139	43.67249
2877.273	32.86637	44.92704	30.16208	23.28246	55.26426	43.93018
2875.344	33.68604	45.33082	29.5438	23.86857	55.70692	43.80978
2873.416	34.40776	45.03233	29.02283	23.84702	56.34014	43.9223

2871.487	34.03445	44.7563	28.80813	24.02135	55.77232	43.8371
2869.559	33.36538	44.38847	29.30738	23.81745	55.63191	43.02719
2867.63	33.09519	44.87179	29.37054	23.93135	55.94262	42.94072
2865.702	33.24666	44.9162	29.48143	24.60347	55.35238	43.18607
2863.773	33.52056	44.29486	29.81354	24.77121	54.91551	43.15248
2861.845	33.36015	44.07705	29.37243	23.90664	55.08047	43.28783
2859.917	33.34157	43.6012	29.1646	23.92728	55.70689	43.33172
2857.988	33.67344	44.04161	29.28347	24.08406	55.68576	43.17927
2856.06	33.54759	44.6183	29.4331	24.03792	56.31252	43.54844
2854.131	33.59171	44.51946	29.62799	24.36156	56.4857	43.52617
2852.203	34.03264	44.77739	29.05678	24.24638	56.11555	44.03391
2850.274	34.1226	44.94581	28.45917	24.39043	55.83361	44.48257
2848.346	34.16028	44.54533	28.67101	24.7726	56.62049	44.91409
2846.417	34.53844	44.43081	29.49773	25.21891	56.63027	44.50638
2844.489	34.3559	44.39597	29.64284	25.19864	56.38314	43.95624
2842.56	34.52645	44.72379	29.53699	24.96119	56.53371	44.09105
2840.632	35.23361	45.34277	30.12866	25.25464	57.78655	45.56104
2838.703	36.08821	45.7685	30.62486	26.15611	58.47779	46.46315
2836.775	36.06099	45.20802	30.60518	25.90411	58.17629	45.83146
2834.846	34.93157	44.9792	30.87574	25.66451	57.89874	45.69138
2832.918	35.1175	45.32718	30.82606	25.66081	57.77413	45.82537
2830.99	35.4386	45.11901	31.02407	26.10487	57.60694	45.78653
2829.061	35.01842	44.80136	31.12951	26.45287	57.16896	45.60993
2827.133	35.06259	44.85736	30.82991	26.05386	56.98849	45.9259
2825.204	35.54298	44.77122	30.86528	25.80493	57.22117	45.9671
2823.276	35.44572	44.75047	30.95265	26.07928	57.1744	45.31209
2821.347	35.36756	44.48986	31.26596	26.20843	57.22718	45.62123
2819.419	35.39095	44.62077	31.19357	25.99876	58.04031	46.19258
2817.49	35.20185	44.79984	31.27667	26.23161	58.26229	46.0002
2815.562	36.19437	45.20127	31.68894	26.1736	57.75169	46.27464
2813.633	36.26258	45.13597	31.92795	26.61213	57.63165	46.74278
2811.705	36.50029	44.85855	31.9835	26.85522	57.63754	46.7573
2809.776	36.64672	44.5778	32.05767	26.66018	57.84994	46.72705
2807.848	36.01502	44.65971	31.73464	26.78534	57.85771	46.15621
2805.919	35.90948	44.78368	31.6043	27.00186	58.14079	46.2314
2803.991	36.0337	44.81497	31.47148	27.08351	58.05139	46.32512
2802.063	35.63223	44.83641	31.68624	26.84383	58.18807	46.35772
2800.134	35.80785	45.09839	32.31467	26.66851	58.33592	46.50542
2798.206	36.65611	45.18178	32.65346	27.0248	59.12949	46.84548
2796.277	36.72153	44.89627	31.99099	27.11776	58.63102	46.64949
2794.349	35.82671	44.96296	31.85237	27.03319	58.13581	46.48121
2792.42	35.83379	45.28734	31.84214	27.29938	58.50033	46.53436
2790.492	36.32863	45.05306	31.66663	27.51263	58.49102	46.45414
2788.563	36.21655	44.32791	31.22312	27.41849	57.95861	45.98335
2786.635	36.2641	44.21468	31.33147	28.16588	58.19328	46.00832
2784.706	35.88046	44.06478	31.6191	27.76451	57.84931	45.97855

2782.778	35.9056	44.15048	31.91769	27.06539	57.46078	46.06791
2780.849	36.56031	44.28541	32.16258	27.21486	57.57568	46.62854
2778.921	36.55435	43.94928	32.07223	27.45725	57.7943	46.74747
2776.992	36.39531	44.15854	32.53912	27.41692	58.46044	46.7296
2775.064	36.77257	44.11197	32.74104	27.66725	58.90371	46.88726
2773.136	36.44193	43.99612	31.98216	27.27917	58.35655	46.70079
2771.207	36.06399	44.23304	32.01381	27.32751	58.16623	46.78047
2769.279	36.29791	44.08351	32.67318	27.73552	57.69368	46.29956
2767.35	36.57131	44.09461	32.3078	27.6034	57.63626	46.35556
2765.422	36.36307	43.74548	31.77167	27.7888	57.70677	46.26167
2763.493	36.19758	43.53953	31.84634	27.97898	57.93212	45.97657
2761.565	36.36288	43.20559	31.52883	27.78397	58.00388	46.1343
2759.636	36.7234	43.58461	31.79941	28.00288	58.24496	46.38552
2757.708	36.98044	44.23421	32.03593	28.20361	58.17846	46.65796
2755.779	36.92889	44.22533	32.07384	28.23664	58.55506	46.81304
2753.851	36.57235	43.89491	32.15889	27.73862	58.30604	46.4173
2751.922	36.75256	43.58905	32.19378	28.00924	58.2413	46.41233
2749.994	36.76916	43.32611	32.28662	28.24842	58.16049	46.44825
2748.065	36.92401	43.48134	32.40014	28.47565	58.30217	46.6932
2746.137	37.0757	43.66924	32.45107	28.75527	58.32539	46.41704
2744.209	36.89161	43.31568	32.20636	28.53091	57.65844	45.63372
2742.28	36.59405	43.03588	32.04897	28.20127	57.60369	45.77026
2740.352	36.73217	43.32408	32.32558	28.66487	58.30614	46.84052
2738.423	36.50524	43.25101	31.98794	28.91071	58.28495	47.09558
2736.495	36.53264	43.10691	31.79869	28.95422	57.953	46.79461
2734.566	36.71503	43.20333	31.74708	28.39665	57.85494	45.80698
2732.638	36.98883	42.92177	31.82047	28.5678	57.94851	45.74294
2730.709	37.05886	42.54585	32.1952	28.46615	57.91222	45.75367
2728.781	37.14912	42.84694	32.47555	28.7287	58.35147	45.70533
2726.852	36.83538	43.24489	32.17754	29.15871	58.17532	45.90298
2724.924	37.05503	42.7709	31.81212	29.15478	58.04317	46.42477
2722.995	36.64235	42.33015	31.82787	28.71857	57.96352	46.45781
2721.067	36.69618	42.38472	31.52259	28.85896	57.66618	46.39357
2719.138	36.70602	42.16599	31.56462	28.77113	57.196	45.99743
2717.21	36.93266	41.9726	31.73225	28.84642	57.52723	45.90011
2715.281	37.11663	42.11501	32.02006	28.76145	57.66463	45.66075
2713.353	37.10743	42.29202	32.30755	28.78032	57.90561	45.57204
2711.425	36.91253	42.20351	32.16001	28.84221	57.77283	45.19889
2709.496	37.0732	41.83665	31.93602	29.13157	57.41462	45.2583
2707.568	37.06762	42.3022	31.91785	29.47158	57.49331	45.23357
2705.639	36.87889	42.21774	31.69424	29.30533	57.58219	45.27237
2703.711	36.71805	42.07256	31.80473	28.88815	57.38664	45.77276
2701.782	36.70895	41.8876	32.17717	29.20919	57.38013	46.07043
2699.854	36.5924	41.78185	32.07904	29.08398	57.04192	45.77899
2697.925	36.77858	41.64417	32.01209	29.23859	57.12785	45.7361
2695.997	36.81639	41.48528	31.7787	29.03933	57.0753	45.55306

2694.068	36.67216	41.33525	31.83981	29.29879	57.38654	45.59626
2692.14	36.56982	41.69027	32.06944	29.17787	57.24318	45.49351
2690.211	36.40122	41.54051	32.07106	28.82978	57.30491	45.30157
2688.283	36.49154	41.34814	31.83455	28.87994	57.33492	45.34468
2686.354	36.75632	41.48705	31.91097	29.10611	57.43171	45.21452
2684.426	36.71846	41.52771	32.11712	29.44323	57.26561	45.04712
2682.498	36.67254	41.32055	32.2378	29.39406	57.1755	45.2212
2680.569	36.7419	41.12085	31.92865	29.39538	56.96871	45.36438
2678.641	37.26674	41.30766	31.90123	29.42127	56.95621	45.14022
2676.712	37.26937	41.39885	32.11007	29.46161	56.88523	45.12886
2674.784	37.07531	41.35065	32.15873	29.32946	56.89095	45.37092
2672.855	36.78293	41.11378	32.29175	29.4698	56.54967	45.19423
2670.927	36.52359	41.05525	32.24416	29.73302	56.44342	45.14713
2668.998	36.28698	40.87076	31.93592	29.46819	56.31926	45.02254
2667.07	36.47559	40.92395	31.85491	29.28307	56.65979	45.14285
2665.141	36.58694	40.86213	32.06553	29.62034	56.78765	45.27299
2663.213	36.51019	40.90733	31.73561	29.75484	57.06746	45.12067
2661.284	36.43702	41.07431	31.48279	29.47732	56.64905	44.70249
2659.356	36.93758	41.03454	31.89174	29.45505	56.53434	44.55667
2657.427	37.03349	40.66743	31.94158	29.55608	56.4335	44.79471
2655.499	36.52005	40.43659	31.84835	29.48301	55.99146	44.64729
2653.571	36.29595	40.65185	31.68767	29.21269	55.69882	44.23347
2651.642	36.34891	40.84656	31.85505	29.05885	55.99515	44.34955
2649.714	36.51138	40.82593	31.79472	28.84955	56.11776	44.56673
2647.785	36.69321	40.46588	31.42476	29.09641	56.00013	44.64248
2645.857	36.7144	40.32413	31.56422	29.409	55.77245	44.38601
2643.928	36.74556	40.21833	32.12363	29.78146	56.01788	44.6684
2642	36.28738	40.03576	31.9341	29.68221	56.19036	44.57912
2640.071	36.38282	40.08502	31.8005	29.52202	56.1386	44.42301
2638.143	36.60948	40.0758	31.61147	29.58674	55.70755	44.48455
2636.214	36.62424	40.02468	31.71338	29.90294	55.66666	44.55015
2634.286	36.60564	40.2411	31.91894	29.964	55.98999	44.56223
2632.357	36.98434	40.10332	31.83261	29.89253	56.10525	44.31834
2630.429	36.72835	39.9379	31.87941	29.48903	55.74842	44.17101
2628.5	36.46119	39.67432	31.94489	29.26605	55.58685	43.98047
2626.572	36.50227	39.54226	31.92452	29.34646	55.631	44.08615
2624.644	36.53793	39.81714	31.88396	29.76347	55.83566	44.16397
2622.715	36.65696	40.06334	31.98645	29.6499	55.90493	44.17163
2620.787	36.45757	40.03057	31.94303	29.76929	56.00776	44.14394
2618.858	36.29337	39.82236	31.7786	29.71482	55.69924	43.89151
2616.93	36.42836	39.6126	31.67808	29.64705	55.5902	43.76297
2615.001	36.2984	39.46577	31.72549	29.65218	55.68343	43.90345
2613.073	36.48484	39.25311	31.63157	29.63602	55.7624	43.89744
2611.144	36.74137	39.62587	31.67066	29.73655	55.56972	43.83817
2609.216	36.72396	39.35268	31.63452	29.85553	55.47697	43.96631
2607.287	36.58755	39.06021	31.84055	30.03531	55.84063	43.85762

2605.359	36.28538	39.02141	31.82128	30.15021	55.93257	43.59914
2603.43	36.25489	38.95122	31.51279	29.86139	55.66614	43.53455
2601.502	36.43671	38.72579	31.56006	29.78586	55.36629	43.56517
2599.573	36.16215	38.70268	31.44524	29.7975	55.07368	43.302
2597.645	36.00687	38.71703	31.45308	29.74689	55.23362	43.34397
2595.717	36.34954	38.60136	31.51814	30.12131	55.39712	43.6031
2593.788	36.74791	38.56368	31.5113	30.03259	55.11481	43.61313
2591.86	36.40228	38.77686	31.37357	29.79613	54.90016	43.51581
2589.931	36.36552	38.99143	31.50653	30.03942	55.31461	43.5421
2588.003	36.57695	38.84991	31.52499	30.08144	55.27162	43.46378
2586.074	36.39459	38.34782	31.42562	29.87371	55.01326	43.34574
2584.146	36.22327	37.93004	31.31432	29.71799	55.15097	43.20245
2582.217	36.2777	37.98592	31.20605	29.79522	55.17088	43.14643
2580.289	36.24305	38.05931	31.4943	30.03908	54.89674	43.06839
2578.36	36.01556	38.20028	31.71973	29.99751	55.03415	43.02004
2576.432	36.29338	38.28934	31.83091	29.8817	55.3501	43.25291
2574.503	36.42867	38.19733	31.48059	30.3245	55.35389	43.30816
2572.575	36.09781	38.01698	31.3243	30.17338	55.01419	43.05861
2570.646	36.20454	37.93646	31.30107	30.02625	54.99401	42.83423
2568.718	36.43858	38.21841	31.20388	30.01034	54.89127	42.86364
2566.79	36.39022	38.01346	31.41416	30.0658	55.09132	43.02955
2564.861	36.48012	37.90182	31.48763	30.33131	55.1556	43.1337
2562.933	36.32581	37.70702	31.24606	30.41459	54.98275	42.88504
2561.004	35.98682	37.61113	31.36498	30.38254	54.86597	42.67777
2559.076	36.28853	37.81242	31.5382	30.39085	54.94395	42.48045
2557.147	36.32671	37.85564	31.31013	30.22367	54.81454	42.38311
2555.219	36.32938	37.73486	31.16719	30.02939	54.41656	42.59832
2553.29	36.5293	37.62204	31.26563	30.06805	54.30607	42.70472
2551.362	36.64824	37.63206	31.36798	30.27666	54.66722	42.80887
2549.433	36.43232	37.71171	31.27713	30.12035	54.7282	42.57495
2547.505	36.29072	37.41099	31.27284	29.85014	54.50922	42.14709
2545.576	36.29411	37.34166	31.49991	30.0011	54.36521	42.27439
2543.648	36.24026	37.35375	31.57866	30.23404	54.40884	42.43336
2541.719	36.21501	37.24221	31.55122	30.1378	54.34167	42.60407
2539.791	36.30824	37.16758	31.40568	30.34429	54.22479	42.6417
2537.863	36.2277	37.07572	31.31532	30.35435	54.01785	42.44738
2535.934	36.22048	36.94241	31.28822	30.41847	54.16985	42.38488
2534.006	36.08111	37.03022	31.3987	30.43036	54.26413	42.48321
2532.077	36.08371	37.09877	31.29669	30.33083	54.09838	42.44337
2530.149	36.12457	37.09414	31.27953	30.2684	53.69246	42.14079
2528.22	36.21899	37.02365	31.45167	30.1943	53.89409	42.00181
2526.292	36.29987	37.02926	31.29999	29.99345	54.06533	41.83147
2524.363	36.20801	37.20712	31.25076	30.03528	53.94357	42.00604
2522.435	35.95867	37.1673	31.4928	30.12743	53.80048	42.10684
2520.506	36.00183	36.94569	31.52338	30.15684	54.0386	42.00859
2518.578	36.10621	37.01354	31.23381	30.39388	53.82783	41.92644

2516.649	36.08416	37.17009	31.19362	30.20763	53.74997	41.77724
2514.721	36.28399	37.32384	31.26023	29.95122	54.03788	41.85081
2512.792	36.03968	37.23409	31.38881	30.0423	54.03454	41.81465
2510.864	35.68818	37.02314	31.3101	30.1957	54.01683	41.83182
2508.936	35.81461	36.99426	31.1159	30.37926	53.76841	41.87971
2507.007	35.71626	36.71548	31.10418	30.24438	53.29968	41.7293
2505.079	35.6337	36.36468	31.31603	29.93106	53.2174	41.65535
2503.15	35.67125	36.61707	31.35681	29.7792	53.35032	41.55081
2501.222	35.79515	36.89383	31.17247	29.87505	53.48481	41.63894
2499.293	35.56633	36.7286	30.97795	29.82808	53.47707	41.6908
2497.365	35.5191	36.79063	31.09315	29.8641	53.37454	41.66223
2495.436	35.48016	36.87392	31.2294	29.78984	53.10788	41.61314
2493.508	35.39553	36.56211	31.15577	29.75157	53.09033	41.44475
2491.579	35.4653	36.46043	31.1702	29.89093	52.96167	41.42392
2489.651	35.47783	36.45347	31.14406	29.99468	53.08566	41.48
2487.722	35.36679	36.35355	30.8954	29.90427	53.04382	41.37326
2485.794	35.51293	36.47215	30.94049	29.99753	52.93945	41.20606
2483.865	35.61069	36.28427	31.24854	29.90064	52.73071	41.13108
2481.937	35.41315	36.03743	31.27541	29.85157	52.65907	41.02713
2480.009	35.22361	36.25994	31.32664	29.71583	52.74916	41.06238
2478.08	35.35194	36.30276	31.16983	29.75167	52.9246	41.12056
2476.152	35.55776	36.17048	30.95887	29.76924	52.61514	41.239
2474.223	35.47974	36.19817	30.9534	29.82009	52.37064	41.13947
2472.295	35.2214	36.1804	30.853	29.89628	52.30508	40.92425
2470.366	35.18	36.31157	31.07885	29.928	52.33165	41.02327
2468.438	35.1856	36.25562	31.19903	29.95569	52.08303	40.99171
2466.509	35.25365	36.07168	31.05039	29.75399	52.13061	40.9209
2464.581	35.27301	36.07117	30.75459	29.67449	52.19712	40.91263
2462.652	35.22182	36.06914	30.81688	29.87852	52.19818	40.85094
2460.724	35.18574	35.91436	30.99552	30.02259	52.21073	40.84716
2458.795	35.19919	35.67392	30.79309	30.10417	52.08992	40.7601
2456.867	35.08509	35.55651	30.68153	29.85967	51.97283	40.54792
2454.938	34.93076	35.69321	30.74188	29.78324	51.99459	40.41788
2453.01	34.71016	35.66552	30.85096	29.87948	51.91888	40.3947
2451.082	35.00635	35.62593	30.95704	29.94305	51.85786	40.62269
2449.153	35.06476	35.7079	30.81982	29.96346	51.8867	40.67073
2447.225	34.79632	35.67978	30.70789	29.85446	51.65212	40.45857
2445.296	34.89938	35.39062	30.70779	29.77841	51.57515	40.45211
2443.368	35.17968	35.27581	30.66989	29.827	51.82666	40.40763
2441.439	35.12729	35.40728	30.55932	29.7014	51.76126	40.03315
2439.511	34.79398	35.33689	30.62734	29.80329	51.66593	40.019
2437.582	34.68845	35.03257	30.81041	29.88759	51.42086	39.98032
2435.654	34.74505	34.90401	30.77027	29.9922	51.21936	39.91057
2433.725	34.71684	34.90649	30.83912	30.0037	51.25814	40.03959
2431.797	34.68571	35.02303	30.80938	29.80458	51.3154	39.98442
2429.868	34.52122	35.00379	30.65373	29.66431	50.78399	39.72595

2427.94	34.48494	34.9032	30.69425	29.74873	50.96449	39.65796
2426.011	34.65403	34.8324	30.69786	29.90209	51.31637	39.77414
2424.083	34.71969	34.78851	30.86715	29.78194	51.2961	39.91781
2422.155	34.65567	34.80543	30.97521	29.76436	51.25878	39.76738
2420.226	34.73505	35.01698	30.86474	29.79078	51.24857	39.44709
2418.298	34.75441	35.09539	30.79627	29.79868	51.066	39.54928
2416.369	34.83951	34.97865	30.83308	29.92789	50.97697	39.6408
2414.441	34.46717	34.68763	30.76407	29.93337	50.80771	39.63895
2412.512	34.50898	34.44931	30.65393	29.87356	50.58042	39.58132
2410.584	34.71266	34.56459	30.58734	29.69503	50.6451	39.50528
2408.655	34.69176	34.58101	30.7384	29.74225	50.81317	39.51566
2406.727	34.53361	34.75033	30.83856	29.83791	50.78065	39.63183
2404.798	34.51287	34.60709	30.84864	29.74396	50.54979	39.58209
2402.87	34.60464	34.4001	30.71763	29.564	50.44386	39.46282
2400.941	34.59045	34.48668	30.65064	29.61553	50.57141	39.40942
2399.013	34.51485	34.56783	30.50787	29.81056	50.59258	39.31317
2397.084	34.50613	34.24945	30.35111	30.01365	50.50422	39.15194
2395.156	34.34636	34.25326	30.6062	29.93083	50.30555	39.10883
2393.228	34.30523	34.34985	30.66814	29.82121	50.34658	39.12339
2391.299	34.27741	34.29336	30.53274	29.92125	50.44001	39.24874
2389.371	34.09438	34.39198	30.59894	29.87087	50.50917	39.38895
2387.442	34.12608	34.44353	30.71561	29.71282	50.47592	39.45263
2385.514	34.19141	34.53467	30.94931	29.82051	50.44575	39.48545
2383.585	34.31568	34.59925	30.99365	29.83552	50.43719	39.49147
2381.657	34.63231	34.72787	30.94168	30.09231	50.58175	39.68331
2379.728	34.8454	35.04823	31.19088	30.4615	51.00787	40.05001
2377.8	35.0172	35.27259	31.49848	30.8371	51.60812	40.37025
2375.871	35.29588	35.55577	31.6754	30.90097	52.2453	40.91722
2373.943	35.88286	35.97771	32.26748	31.30937	52.6825	41.36612
2372.014	36.42591	36.63725	32.93615	31.84304	53.21718	41.81019
2370.086	36.65655	36.78671	32.92104	32.20807	53.71925	42.27646
2368.157	36.67257	36.88898	32.85555	32.44846	54.07056	42.71321
2366.229	37.09913	37.03036	33.21991	32.62755	54.8071	43.02139
2364.301	37.61608	37.16473	33.07559	32.5984	54.98416	42.99416
2362.372	37.56516	36.58275	32.83178	32.58683	54.25635	42.78194
2360.444	37.1364	36.0035	32.50174	32.26501	53.38691	42.4847
2358.515	36.93134	35.52988	32.1116	32.22295	52.96247	41.91724
2356.587	36.51954	35.24516	31.7024	31.83204	52.09753	41.44174
2354.658	36.05116	34.76823	31.29684	31.33309	50.83107	40.85287
2352.73	35.08255	34.10234	31.19383	30.55159	49.67899	39.9234
2350.801	34.23752	33.81221	30.96934	29.86433	48.84956	39.41369
2348.873	34.18406	34.86557	31.24179	29.80505	49.52922	39.99748
2346.944	35.22695	36.0752	32.03258	31.05583	52.02929	41.18201
2345.016	35.9434	35.94568	32.32265	31.60999	52.94607	41.28449
2343.087	35.97791	35.51849	32.13675	31.24649	52.59496	41.08468
2341.159	36.05685	35.48959	31.94985	31.20174	52.29401	41.29665

2339.23	36.06484	35.54254	32.11074	31.2604	51.84081	40.89698
2337.302	35.95999	35.31962	32.15653	31.23527	51.18007	40.69543
2335.374	35.73148	34.90425	31.76284	31.02613	50.91349	40.52172
2333.445	35.47281	34.63565	31.68413	30.79588	50.82884	40.33865
2331.517	35.23344	34.5563	31.29671	30.74263	50.75836	40.15348
2329.588	34.9716	34.29409	31.2807	30.67136	50.40924	40.04473
2327.66	34.63202	34.1627	31.26015	30.43041	49.92549	39.88427
2325.731	34.5116	34.04187	31.00172	30.24596	49.75385	39.34758
2323.803	34.46391	33.9101	30.96765	30.19826	50.0955	39.20643
2321.874	34.31514	34.11104	30.96656	29.89379	49.77001	39.0314
2319.946	34.02176	34.06529	30.7375	29.89147	49.30285	38.5521
2318.017	33.93378	33.74347	30.48659	29.79526	48.93546	38.41152
2316.089	33.84981	33.45869	30.50748	29.83991	48.69761	38.58339
2314.16	33.71152	33.40641	30.39668	29.81042	48.75913	38.6224
2312.232	33.72619	33.27223	30.56663	29.76038	48.73186	38.48472
2310.303	33.64146	33.20677	30.54658	29.56291	48.36611	38.10149
2308.375	33.54797	33.1881	30.30778	29.52279	48.43693	38.05153
2306.447	33.37736	33.16417	30.08476	29.4475	48.49493	38.04765
2304.518	33.5224	33.18917	30.13847	29.5538	48.42507	38.1696
2302.59	33.61987	33.21735	30.36719	29.53873	48.44932	38.30012
2300.661	33.56842	33.13557	30.41185	29.51892	48.45518	38.21646
2298.733	33.65396	33.10533	30.26228	29.38696	48.34747	37.99636
2296.804	33.62569	33.12799	30.19366	29.47283	48.3948	37.80553
2294.876	33.54166	33.10607	30.29918	29.45353	48.40894	37.82088
2292.947	33.48703	32.91174	30.2492	29.4814	48.39167	37.76817
2291.019	33.41465	32.97288	30.19138	29.55304	48.29673	37.82988
2289.09	33.48833	32.89011	30.25245	29.59993	48.32724	37.93756
2287.162	33.52385	32.96809	30.19148	29.61426	48.36129	37.87356
2285.233	33.5243	33.07496	30.20446	29.57593	48.3165	37.84732
2283.305	33.59438	33.09453	30.38551	29.64559	48.29474	37.68028
2281.376	33.55186	32.89037	30.34528	29.68274	48.33115	37.58806
2279.448	33.4733	32.83437	30.34087	29.55235	48.44809	37.65067
2277.52	33.55454	33.02019	30.27173	29.5778	48.41596	37.75386
2275.591	33.54841	33.09375	30.29116	29.7061	48.41847	37.74545
2273.663	33.48373	32.96169	30.42429	29.66313	48.41695	37.82911
2271.734	33.49065	32.98045	30.3838	29.53302	48.39171	37.83452
2269.806	33.42413	32.9071	30.22764	29.59832	48.35204	37.69343
2267.877	33.35186	32.85411	30.19908	29.65605	48.2078	37.61781
2265.949	33.37541	32.89621	30.17352	29.67414	48.15767	37.58964
2264.02	33.24121	32.82384	30.28455	29.55242	48.18197	37.50282
2262.092	33.28016	32.79821	30.33856	29.67072	48.18684	37.48401
2260.163	33.42341	32.8355	30.31569	29.67668	48.04884	37.62606
2258.235	33.48408	32.74015	30.24887	29.71374	48.11777	37.58145
2256.306	33.44076	32.70742	30.1646	29.66501	48.1764	37.42572
2254.378	33.49792	32.73612	30.30957	29.55474	48.13727	37.43761
2252.449	33.47324	32.69767	30.4188	29.49563	48.06836	37.4476

2250.521	33.45696	32.59053	30.32349	29.60424	47.89735	37.44912
2248.593	33.41173	32.60442	30.25952	29.64311	47.94462	37.36391
2246.664	33.43524	32.62071	30.25576	29.69072	47.91904	37.38111
2244.736	33.47419	32.63184	30.28951	29.62477	47.71886	37.34004
2242.807	33.41341	32.61004	30.36024	29.4812	47.7141	37.22307
2240.879	33.38379	32.60831	30.40694	29.51946	47.8765	37.32193
2238.95	33.40167	32.46803	30.30349	29.69736	47.90072	37.29845
2237.022	33.32665	32.40478	30.2153	29.63689	47.84506	37.18241
2235.093	33.23322	32.50927	30.22716	29.60661	47.74383	37.24749
2233.165	33.22978	32.51905	30.29071	29.55555	47.70807	37.21275
2231.236	33.32466	32.52358	30.27876	29.56368	47.71873	37.20364
2229.308	33.24005	32.57683	30.33638	29.60527	47.78483	37.31191
2227.379	33.14121	32.57659	30.26995	29.53318	47.77408	37.34997
2225.451	33.16203	32.60498	30.25322	29.59119	47.62035	37.21675
2223.522	33.24036	32.60923	30.29326	29.69686	47.49121	37.14641
2221.594	33.20432	32.50626	30.41712	29.67887	47.59096	37.30587
2219.666	33.18906	32.44344	30.38422	29.63286	47.55723	37.11876
2217.737	33.12165	32.41688	30.29976	29.50173	47.44285	36.97283
2215.809	33.08461	32.37989	30.27277	29.46752	47.50621	36.96611
2213.88	33.11634	32.43056	30.33141	29.50679	47.62582	36.94138
2211.952	33.19016	32.44688	30.35286	29.61855	47.45695	36.9142
2210.023	33.02532	32.40202	30.30092	29.56874	47.32907	37.00201
2208.095	32.92941	32.40153	30.36023	29.59085	47.38651	37.04216
2206.166	32.96007	32.34691	30.2703	29.66439	47.32157	36.9935
2204.238	33.02872	32.46825	30.24776	29.60941	47.14357	36.98843
2202.309	32.99597	32.4437	30.36851	29.51397	47.07647	36.99672
2200.381	32.98901	32.19095	30.30613	29.48041	47.06271	36.95621
2198.452	32.98103	32.17093	30.22136	29.46605	47.00896	36.89367
2196.524	32.96611	32.1856	30.22832	29.48956	47.00409	36.86249
2194.595	32.96219	32.2175	30.2889	29.46878	46.99497	36.94655
2192.667	32.89033	32.30639	30.42638	29.54298	47.04007	36.94113
2190.739	32.8701	32.29811	30.42822	29.48219	46.99549	36.90029
2188.81	32.79938	32.21196	30.35955	29.41312	46.96892	36.84688
2186.882	32.82015	32.23435	30.34346	29.5019	46.82136	36.69569
2184.953	32.82929	32.28833	30.22828	29.47006	46.72794	36.69953
2183.025	32.83334	32.25554	30.23413	29.40585	46.84666	36.68413
2181.096	32.70015	32.18967	30.30693	29.42942	46.83924	36.72394
2179.168	32.7476	32.22371	30.14442	29.39893	46.80712	36.65982
2177.239	32.84157	32.22034	30.25351	29.42361	46.71402	36.59274
2175.311	32.82712	32.19343	30.28894	29.41053	46.66364	36.60625
2173.382	32.83614	32.21442	30.29019	29.34981	46.7553	36.70569
2171.454	32.75505	32.161	30.24008	29.34116	46.69276	36.65102
2169.525	32.72334	32.19417	30.12262	29.41703	46.62614	36.54831
2167.597	32.71047	32.24575	30.12881	29.32846	46.63297	36.61674
2165.668	32.64756	32.11991	30.27664	29.29068	46.61594	36.64522
2163.74	32.61835	32.04328	30.32857	29.38548	46.6099	36.67448

2161.812	32.65756	32.08767	30.31276	29.40545	46.62521	36.56456
2159.883	32.6315	32.06525	30.22869	29.37108	46.5336	36.39719
2157.955	32.70521	32.0704	30.21154	29.34811	46.43492	36.25979
2156.026	32.59499	32.01443	30.20169	29.31419	46.39629	36.29122
2154.098	32.52295	31.88853	30.22335	29.45895	46.40231	36.37098
2152.169	32.56785	31.88253	30.2893	29.47991	46.39556	36.35833
2150.241	32.65177	31.98034	30.28816	29.31532	46.35292	36.27272
2148.312	32.54541	31.97765	30.36016	29.30019	46.3605	36.27436
2146.384	32.5316	31.95267	30.30188	29.4268	46.32871	36.33759
2144.455	32.55808	31.99653	30.2314	29.35545	46.3299	36.35394
2142.527	32.6244	31.9461	30.26307	29.26968	46.28273	36.38604
2140.598	32.52182	31.82589	30.22305	29.27069	46.11991	36.26799
2138.67	32.43361	31.87419	30.20455	29.3387	46.13084	36.29115
2136.741	32.36472	31.95816	30.2178	29.25332	46.17351	36.32115
2134.813	32.30261	31.91217	30.27057	29.20353	46.16997	36.29145
2132.885	32.43334	31.90878	30.24487	29.26523	46.10165	36.1785
2130.956	32.57191	31.94205	30.1901	29.35696	46.08033	36.11869
2129.028	32.50695	31.91214	30.18358	29.41068	46.02631	36.19726
2127.099	32.44569	31.80476	30.20957	29.43556	45.935	36.17126
2125.171	32.44419	31.78518	30.19192	29.47413	45.96819	36.12244
2123.242	32.42381	31.69095	30.25804	29.43366	46.03371	36.01702
2121.314	32.37598	31.71049	30.27508	29.3616	45.9404	35.91901
2119.385	32.44965	31.7922	30.23206	29.48956	45.90831	36.04202
2117.457	32.50368	31.81932	30.1305	29.43342	45.8961	36.07447
2115.528	32.29983	31.71921	30.17581	29.38825	45.84569	35.92988
2113.6	32.30442	31.6352	30.25802	29.38341	45.73708	35.81379
2111.671	32.3061	31.64904	30.24368	29.25491	45.64963	35.81175
2109.743	32.24356	31.6912	30.22002	29.31246	45.67041	35.89936
2107.814	32.32931	31.68303	30.19587	29.29879	45.62025	35.89105
2105.886	32.39036	31.62961	30.20083	29.25386	45.57104	35.86035
2103.958	32.45066	31.64915	30.16798	29.32862	45.69101	35.83454
2102.029	32.53482	31.72964	30.18694	29.41235	45.80306	35.84753
2100.101	32.42496	31.62432	30.30619	29.41624	45.80052	35.80547
2098.172	32.31723	31.56431	30.28996	29.36563	45.77829	35.83218
2096.244	32.34041	31.71124	30.23884	29.35822	45.65627	35.80582
2094.315	32.21765	31.78662	30.17827	29.40522	45.61058	35.84895
2092.387	32.17012	31.60026	30.16381	29.41578	45.58594	35.90303
2090.458	32.1642	31.48565	30.19279	29.40104	45.46716	35.75259
2088.53	32.26646	31.52951	30.21786	29.47083	45.48222	35.66936
2086.601	32.28775	31.62119	30.31918	29.44048	45.58971	35.65763
2084.673	32.32273	31.58606	30.36236	29.47409	45.56767	35.74044
2082.744	32.24754	31.50533	30.32483	29.42829	45.42516	35.74152
2080.816	32.16542	31.53275	30.23754	29.40193	45.40147	35.71741
2078.887	32.28988	31.54004	30.32883	29.42248	45.5043	35.78225
2076.959	32.3423	31.56128	30.30648	29.4384	45.51143	35.71801
2075.031	32.3015	31.62921	30.30866	29.46957	45.49628	35.68129

2073.102	32.23954	31.53957	30.30801	29.36593	45.33885	35.69073
2071.174	32.17948	31.50844	30.27179	29.40477	45.31496	35.67248
2069.245	32.20536	31.52111	30.26653	29.46123	45.35128	35.68707
2067.317	32.20041	31.52988	30.25994	29.34204	45.33938	35.62096
2065.388	32.13653	31.53638	30.28856	29.19623	45.22304	35.36022
2063.46	32.11768	31.60749	30.38029	29.2526	45.1227	35.41302
2061.531	32.23026	31.66405	30.40119	29.40557	45.30829	35.63605
2059.603	32.22276	31.60217	30.36905	29.49078	45.36937	35.61311
2057.674	32.23201	31.56854	30.37476	29.43733	45.32951	35.59988
2055.746	32.12588	31.46395	30.22703	29.39522	45.25077	35.57471
2053.817	32.15154	31.45329	30.13962	29.34889	45.228	35.49369
2051.889	32.11029	31.48965	30.24358	29.3386	45.23739	35.53073
2049.96	32.07195	31.48351	30.36304	29.38209	45.24956	35.59548
2048.032	32.12736	31.55151	30.36819	29.42129	45.18518	35.59716
2046.103	32.19479	31.54489	30.32386	29.43182	45.07604	35.54328
2044.175	32.15741	31.45572	30.25511	29.37971	44.99825	35.54049
2042.246	32.08715	31.36596	30.2489	29.35034	44.95683	35.39777
2040.318	32.04498	31.36272	30.2553	29.32657	44.87808	35.29354
2038.39	32.15637	31.51046	30.36974	29.35312	44.97284	35.4445
2036.461	32.2388	31.66381	30.44525	29.39099	45.03775	35.48914
2034.533	32.18753	31.60966	30.33608	29.3669	45.03677	35.52123
2032.604	32.09282	31.52908	30.34603	29.29501	45.1152	35.60748
2030.676	32.14522	31.56452	30.36492	29.25945	45.12041	35.524
2028.747	32.18631	31.54636	30.40612	29.37398	45.17006	35.46859
2026.819	32.13987	31.51597	30.43776	29.46204	45.05748	35.49064
2024.89	32.10677	31.54268	30.43665	29.45525	44.91864	35.50755
2022.962	32.16363	31.60815	30.4215	29.39486	44.87759	35.49621
2021.033	32.15713	31.69526	30.46943	29.4037	44.9548	35.50453
2019.105	32.09563	31.47012	30.46767	29.36694	44.9282	35.42161
2017.176	31.95902	31.21737	30.37929	29.21946	44.66993	35.1434
2015.248	32.06889	31.35675	30.36076	29.21741	44.57416	35.25798
2013.319	32.19522	31.58208	30.45007	29.37858	44.83791	35.44712
2011.391	32.12082	31.60031	30.50263	29.42037	44.90755	35.44436
2009.463	32.07484	31.55618	30.49542	29.36788	44.88434	35.43979
2007.534	32.14089	31.53813	30.47149	29.34501	44.76591	35.42402
2005.606	32.09541	31.62593	30.49737	29.41886	44.79966	35.50776
2003.677	32.08234	31.61821	30.56491	29.41207	44.8251	35.38022
2001.749	32.14911	31.65278	30.58256	29.44885	44.87137	35.38881
1999.82	32.18227	31.63961	30.60403	29.45924	44.83472	35.39665
1997.892	32.23364	31.60932	30.67171	29.48273	44.87885	35.42753
1995.963	32.30346	31.62373	30.72056	29.49781	44.96375	35.41222
1994.035	32.17316	31.60544	30.78464	29.5096	44.97541	35.29082
1992.106	31.89633	31.27859	30.65343	29.34913	44.50386	34.77413
1990.178	32.0248	31.3346	30.51447	29.24869	44.26178	35.00659
1988.249	32.07509	31.47051	30.6282	29.34335	44.5257	35.19738
1986.321	32.06434	31.52473	30.70672	29.34113	44.63872	35.27498

1984.392	32.14027	31.63309	30.75318	29.39751	44.76136	35.36481
1982.464	32.1603	31.65315	30.75759	29.46501	44.72221	35.28803
1980.536	32.2389	31.62564	30.86191	29.53338	44.69547	35.31573
1978.607	32.2415	31.56901	30.8979	29.49819	44.68896	35.30915
1976.679	32.18398	31.57642	30.91358	29.49943	44.67804	35.31443
1974.75	32.15223	31.6306	30.91195	29.50178	44.736	35.33901
1972.822	32.23122	31.75148	30.89632	29.5669	44.83281	35.36885
1970.893	32.31008	31.74166	30.8716	29.50698	44.77396	35.30647
1968.965	32.22422	31.6483	30.87662	29.51953	44.70301	35.16418
1967.036	31.8871	31.35667	30.68619	29.41886	44.3279	34.80451
1965.108	32.08948	31.4348	30.79101	29.51802	44.33274	35.08476
1963.179	32.35296	31.74716	31.03213	29.64589	44.7342	35.34812
1961.251	32.33573	31.67044	30.97552	29.58939	44.73462	35.31716
1959.322	32.38392	31.64599	30.92392	29.5878	44.72937	35.40381
1957.394	32.4057	31.66519	30.96284	29.63264	44.71333	35.35014
1955.465	32.30012	31.67042	30.98352	29.66498	44.65101	35.2338
1953.537	32.30668	31.66024	30.9537	29.72292	44.57008	35.30083
1951.609	32.34528	31.73175	31.01289	29.72808	44.6566	35.41167
1949.68	32.35723	31.75642	31.1042	29.794	44.66169	35.34679
1947.752	32.30783	31.77519	31.15754	29.78526	44.7152	35.2995
1945.823	32.21795	31.60157	31.083	29.75588	44.64883	35.12971
1943.895	32.00853	31.41847	30.91338	29.6305	44.46785	34.88659
1941.966	31.76261	31.04031	30.62892	29.30412	43.77672	34.49962
1940.038	32.19931	31.45154	30.93724	29.51024	44.08801	35.19588
1938.109	32.40305	31.67033	31.15199	29.85276	44.73543	35.39066
1936.181	32.37738	31.67646	31.13006	29.87383	44.68615	35.32325
1934.252	32.32105	31.68548	31.12076	29.83104	44.59529	35.29438
1932.324	32.39202	31.80384	31.16637	29.79321	44.64785	35.34509
1930.395	32.5127	31.80862	31.23265	29.86927	44.7189	35.35987
1928.467	32.54498	31.82267	31.1681	29.8464	44.65302	35.39064
1926.538	32.47644	31.86059	31.17503	29.86642	44.66972	35.39381
1924.61	32.28071	31.77568	31.21762	29.80021	44.5901	35.25254
1922.682	31.87697	31.24215	30.94392	29.61572	43.96407	34.65964
1920.753	32.06131	31.48719	31.08312	29.65475	44.04673	35.14302
1918.825	32.14671	31.60228	31.19457	29.76649	44.39087	34.88699
1916.896	32.0017	31.33235	30.94823	29.60394	43.8104	34.70153
1914.968	32.34987	31.67396	31.19036	29.78347	44.30605	35.48338
1913.039	32.41944	31.84735	31.32515	29.90528	44.65914	35.42715
1911.111	32.25948	31.74181	31.30103	29.87543	44.52575	35.22437
1909.182	32.0198	31.50484	31.12902	29.79022	44.11443	34.90079
1907.254	32.2378	31.70426	31.21169	29.90742	44.16745	35.14534
1905.325	32.44301	31.89643	31.33543	29.95612	44.58376	35.35402
1903.397	32.46559	31.84833	31.34861	29.9196	44.66638	35.37181
1901.468	32.4874	31.84641	31.31917	29.88914	44.53243	35.37016
1899.54	32.50673	31.88502	31.32879	29.91887	44.52024	35.44077
1897.611	32.42254	31.85907	31.3639	29.93976	44.5639	35.47866

1895.683	32.17879	31.67022	31.29725	29.80818	44.34507	35.11076
1893.755	32.20614	31.58893	31.18407	29.65404	44.04553	35.12548
1891.826	32.47034	31.88705	31.42438	29.86628	44.42367	35.43875
1889.898	32.08681	31.65876	31.30143	29.78156	44.16546	34.77796
1887.969	32.14446	31.52463	31.19723	29.73943	43.71835	34.94038
1886.041	32.42128	31.90368	31.40262	30.0167	44.2449	35.42569
1884.112	32.35208	31.91323	31.33934	29.95365	44.28922	35.2307
1882.184	32.4437	31.92651	31.40007	29.96216	44.34305	35.42849
1880.255	32.4699	31.94288	31.42419	29.99137	44.42437	35.4001
1878.327	32.43584	31.86875	31.39623	29.97846	44.43286	35.36308
1876.398	32.47377	31.92271	31.48429	29.99605	44.39256	35.39675
1874.47	32.4706	32.05113	31.56757	30.00272	44.46857	35.45971
1872.541	32.40861	32.08416	31.60028	29.95294	44.48877	35.41627
1870.613	32.0112	31.74598	31.4441	29.87055	44.25622	34.86377
1868.684	31.17287	30.8334	30.86176	29.45904	42.9229	33.6637
1866.756	31.52815	31.08398	30.9674	29.41551	42.62693	34.38902
1864.828	32.27586	31.78263	31.46239	29.85961	43.79573	35.36212
1862.899	32.39908	31.96926	31.55177	29.99243	44.27888	35.48001
1860.971	32.27768	31.83861	31.50593	29.88914	44.13223	35.21584
1859.042	32.25996	31.85359	31.53583	29.88823	44.14177	35.23619
1857.114	32.33873	31.96809	31.57688	29.9791	44.26699	35.30719
1855.185	32.44032	32.0012	31.60919	30.02574	44.34732	35.41769
1853.257	32.46704	32.00881	31.63155	30.00241	44.39766	35.40942
1851.328	32.44138	32.03518	31.63376	30.00136	44.35871	35.55113
1849.4	32.20495	31.9193	31.5862	29.98252	44.24157	35.25518
1847.471	31.85518	31.66539	31.50479	29.9136	43.86514	34.99898
1845.543	31.53025	31.3968	31.36699	29.87523	43.66973	34.43511
1843.614	31.16485	30.86931	30.81644	29.34235	42.52661	33.59406
1841.686	31.84821	31.60384	31.29759	29.54627	42.96677	35.01429
1839.757	32.37167	32.19323	31.82596	30.12293	44.12127	35.60611
1837.829	32.1972	31.93782	31.67649	30.04788	43.9658	35.16222
1835.901	32.19604	31.87179	31.62383	30.02871	43.90157	35.23502
1833.972	32.31065	31.95894	31.73388	30.04302	44.03706	35.40446
1832.044	32.16986	31.99	31.82541	30.09693	44.24109	35.20248
1830.115	31.35973	31.18171	31.19162	29.66706	43.32637	33.83358
1828.187	31.59848	31.24763	31.07269	29.46264	42.64551	34.39
1826.258	32.01249	31.7823	31.63492	29.93005	43.64369	35.00368
1824.33	31.59998	31.33078	31.37995	29.66913	43.11686	34.37589
1822.401	32.14737	31.76236	31.67332	29.87709	43.4131	35.11798
1820.473	32.37922	31.99157	31.83017	30.13941	44.0341	35.2243
1818.544	32.28376	31.94303	31.76412	30.06855	43.97395	35.05845
1816.616	32.25737	31.88227	31.70239	30.08773	43.87171	34.83256
1814.687	32.40776	32.00658	31.79555	30.18188	43.95467	35.09212
1812.759	32.13792	31.87593	31.78116	30.08133	43.92982	34.75601
1810.83	31.7427	31.45672	31.49419	29.77834	43.31876	34.16002
1808.902	31.97598	31.54164	31.5608	29.8246	43.39715	34.4207

1806.974	32.25491	31.83566	31.74114	30.02364	43.73979	34.73991
1805.045	32.27868	31.98378	31.85934	30.14421	43.99735	34.73149
1803.117	31.98204	31.75657	31.75874	30.10544	43.73747	34.31964
1801.188	31.43517	31.27734	31.4328	29.81835	43.0591	33.48934
1799.26	31.64869	31.36548	31.4124	29.70362	42.84459	33.66386
1797.331	31.87861	31.69674	31.69424	29.97408	43.25766	34.00939
1795.403	31.78576	31.71525	31.75801	30.01188	43.27653	33.77476
1793.474	31.43892	31.23853	31.52527	29.87678	43.1439	32.92822
1791.546	30.92187	30.47885	30.84076	29.30203	41.80881	32.08918
1789.617	31.87606	31.33816	31.4821	29.73038	42.52217	33.49426
1787.689	32.40938	32.01269	31.99991	30.22434	43.77229	34.06947
1785.76	32.00999	31.71365	31.8249	30.13177	43.65775	33.28805
1783.832	31.82492	31.53696	31.55006	29.95326	43.11168	32.96107
1781.903	31.9486	31.71276	31.74009	30.00809	43.37519	32.99047
1779.975	31.50886	31.23914	31.51423	29.80675	42.72653	32.33554
1778.047	31.88938	31.64636	31.79056	30.08133	43.10912	32.93987
1776.118	31.8799	31.79617	31.84948	30.1892	43.4756	32.80876
1774.19	31.40848	31.45849	31.57206	29.96159	43.1432	32.15155
1772.261	30.38667	30.38856	30.79644	29.33906	41.78671	30.49192
1770.333	30.98988	30.76231	31.03491	29.43156	41.47639	31.50469
1768.404	31.61965	31.27408	31.48719	29.92583	42.6608	31.98031
1766.476	31.79558	31.39392	31.47817	29.89873	42.72231	32.04586
1764.547	32.16681	31.94842	31.97438	30.29095	43.62559	32.52951
1762.619	31.42746	31.3103	31.61138	29.95622	43.15054	31.30452
1760.69	31.27374	30.94587	31.27	29.62671	42.15823	31.18429
1758.762	31.73225	31.51947	31.70506	29.99784	42.89962	31.86397
1756.833	31.36916	31.18618	31.4304	29.80386	42.72469	31.0063
1754.905	31.72571	31.43477	31.43107	29.79505	42.6221	31.38931
1752.976	31.63427	31.60642	31.65026	30.07116	43.21589	31.19535
1751.048	30.22736	30.33682	30.76397	29.33244	41.66489	29.35639
1749.12	30.02468	29.99521	30.5879	29.08098	40.72766	29.25505
1747.191	30.38408	30.34322	30.79224	29.21151	40.93254	29.63907
1745.263	30.92356	30.79898	31.10615	29.54247	41.693	30.07499
1743.334	30.8082	30.65337	30.98092	29.44114	41.75226	29.54786
1741.406	31.05584	30.87941	31.01807	29.54483	42.08204	29.72728
1739.477	30.67416	30.51725	30.64728	29.39741	41.81182	29.13137
1737.549	31.09329	30.91471	30.88416	29.51937	41.87614	29.90632
1735.62	30.64093	30.81782	30.97363	29.687	42.519	29.02274
1733.692	28.62603	28.54816	29.20464	28.12606	39.34624	26.55915
1731.763	30.03527	29.80499	30.13998	28.79245	40.1695	28.89433
1729.835	30.68797	30.48092	30.5758	29.27294	41.40868	29.26588
1727.906	31.12419	30.74038	30.66282	29.38187	41.90834	29.59913
1725.978	31.31334	31.02919	30.88017	29.65981	42.53035	29.80624
1724.049	31.00563	30.76719	30.69614	29.45293	42.209	29.32741
1722.121	30.99186	30.70517	30.70705	29.30167	41.96943	29.4418
1720.193	30.82952	30.73808	30.92895	29.49006	42.32351	29.37449

1718.264	29.04709	29.08456	29.75865	28.59226	40.47162	27.08039
1716.336	28.55141	28.29733	29.12787	27.85242	38.44038	26.90143
1714.407	29.57508	29.41382	29.91074	28.52699	39.70793	28.29137
1712.479	30.49041	30.33109	30.51042	28.98817	41.03661	29.31493
1710.55	30.72608	30.681	30.65074	29.20579	41.81131	29.51778
1708.622	30.43663	30.47121	30.432	29.08243	41.73024	29.2373
1706.693	29.85486	29.96426	30.30509	28.89586	41.29673	28.57673
1704.765	29.32323	29.23741	29.79349	28.38804	40.0499	28.09516
1702.836	29.96621	30.04784	30.51102	29.01687	41.04462	29.29428
1700.908	28.56635	28.79702	29.65367	28.51866	40.48279	27.06993
1698.979	28.09114	27.9507	28.80331	27.50329	38.0971	27.26415
1697.051	28.78483	29.01257	29.86006	28.47738	39.74798	28.21519
1695.122	28.52736	28.4644	29.24423	28.02975	38.92842	27.47031
1693.194	29.94844	29.86495	30.22248	28.713	40.59516	29.6437
1691.266	29.81606	30.05088	30.3796	28.90207	41.48257	29.45543
1689.337	28.84509	29.17099	29.70871	28.25117	40.33915	28.42667
1687.409	28.66955	29.01907	29.72813	28.23194	40.10861	28.53645
1685.48	27.73413	28.43221	29.33137	28.13505	39.82862	27.12937
1683.552	26.06552	26.43833	27.51882	26.53252	36.39787	25.24564
1681.623	28.09131	28.3095	29.07822	27.63288	38.513	28.48685
1679.695	28.36736	28.72417	29.45939	27.93427	39.72705	28.47843
1677.766	28.46621	28.86845	29.57661	27.92253	40.16983	28.69172
1675.838	27.57158	28.21956	29.03844	27.4863	39.77922	27.41881
1673.909	27.33274	27.79394	28.60958	26.99285	38.63115	27.554
1671.981	27.51122	28.14576	29.04998	27.4581	39.48146	27.95194
1670.052	26.27913	26.97486	28.13447	26.62177	38.06993	26.185
1668.124	26.44066	26.94576	27.95236	26.4721	37.56084	26.58296
1666.195	27.43988	27.86907	28.59573	27.1144	39.08582	27.93898
1664.267	27.0043	27.69033	28.49526	27.04228	39.49088	27.29828
1662.339	25.95157	26.66759	27.61738	26.23529	37.85258	26.01613
1660.41	26.44036	27.1052	27.99189	26.53695	38.16742	26.85439
1658.482	26.52139	27.30177	28.10903	26.67568	38.75556	26.95448
1656.553	25.93084	26.9469	27.86394	26.43575	38.56417	26.28494
1654.625	24.19459	25.55351	26.90659	25.62961	37.08658	23.89758
1652.696	22.36508	23.10541	24.86424	23.80034	33.26163	21.38826
1650.768	25.02303	25.63348	26.83859	25.41494	35.95528	25.61572
1648.839	24.80288	25.9514	27.24518	25.81973	37.37195	25.35818
1646.911	22.83836	24.04084	25.57815	24.25051	34.76171	22.90929
1644.982	23.75896	24.67058	25.66445	24.50297	35.12885	24.44658
1643.054	24.49892	25.53645	25.95277	24.99709	36.72713	25.48502
1641.125	24.04111	25.29825	25.31804	24.81348	36.93409	25.16286
1639.197	23.43052	24.75443	24.49208	24.4185	36.44667	24.63846
1637.268	22.42972	24.01242	23.82277	23.93301	35.54666	23.3938
1635.34	21.53908	22.93148	22.9684	23.0243	33.50384	22.24759
1633.412	23.35712	24.39769	24.29635	24.03785	35.04238	24.75477
1631.483	23.79783	24.97999	24.82751	24.51089	36.2491	25.18599

1629.555	23.33521	24.64016	24.52519	24.26653	36.057	24.47415
1627.626	22.79618	24.02189	23.79159	23.78786	35.11842	23.89841
1625.698	22.93634	24.24236	23.62653	23.91145	35.30303	24.32247
1623.769	22.28267	23.70053	22.7636	23.57082	34.79381	23.46243
1621.841	22.37604	23.55773	21.9612	23.27116	34.45932	23.8399
1619.912	22.81185	24.08989	21.84067	23.61087	35.54151	24.65142
1617.984	22.1542	23.63435	21.11857	23.30418	35.2521	23.67335
1616.055	21.79883	23.1624	20.78086	22.87713	33.97458	23.12946
1614.127	23.2279	24.45973	22.29492	23.8188	35.362	24.988
1612.198	23.69775	25.01732	23.3528	24.26043	36.31652	25.11082
1610.27	23.83647	25.08805	23.97079	24.29143	36.23748	24.79436
1608.341	23.96812	25.24134	24.49058	24.33175	36.13924	24.65536
1606.413	24.38349	25.61847	25.08617	24.5716	36.45068	25.09536
1604.485	24.66997	25.90604	25.62192	24.89139	36.87403	25.39181
1602.556	24.88338	26.10104	25.93052	25.10317	36.9922	25.53035
1600.628	25.30815	26.44599	26.27535	25.35063	37.35168	25.86015
1598.699	25.7628	26.86632	26.61791	25.68461	37.7841	26.41833
1596.771	25.9047	27.01653	26.79224	25.85924	37.88513	26.52391
1594.842	26.07858	27.14665	27.01007	26.02261	37.91998	26.66413
1592.914	26.37301	27.41004	27.32504	26.27656	38.23933	26.98928
1590.985	26.69694	27.7535	27.77812	26.62255	38.66186	27.30372
1589.057	27.01465	27.9862	28.1386	26.85963	38.89482	27.5078
1587.128	27.54737	28.33275	28.55313	27.15046	39.29683	27.97075
1585.2	27.73729	28.51494	28.73738	27.29184	39.51593	28.13879
1583.271	28.05311	28.77047	28.94527	27.58419	39.73811	28.46961
1581.343	28.3223	29.05303	29.12647	27.86654	39.97256	28.6703
1579.414	28.53752	29.29976	29.39869	28.13941	40.20787	28.93474
1577.486	27.66949	28.67888	29.0777	27.88942	39.60537	27.74883
1575.558	27.54101	28.32491	28.67963	27.51389	38.38535	27.53565
1573.629	29.28395	29.84882	29.9507	28.5843	40.13922	29.88731
1571.701	29.40705	30.15061	30.2096	29.01813	41.06691	29.78973
1569.772	28.16411	28.99209	29.12749	28.27818	39.50358	28.13473
1567.844	29.21191	29.74849	29.55232	28.7901	39.90412	29.74774
1565.915	29.90842	30.42352	30.03611	29.38798	41.30569	30.42194
1563.987	29.7652	30.27287	29.55124	29.14709	41.03372	30.16453
1562.058	29.87063	30.59374	29.61054	29.49263	41.45156	30.31254
1560.13	27.46321	28.74029	28.11743	28.50848	39.54596	27.0839
1558.201	26.09178	26.67949	26.44681	26.5929	34.98188	25.52097
1556.273	29.8779	30.3125	29.42912	29.12068	39.47467	30.70214
1554.344	29.93407	30.58116	29.78597	29.52726	40.57941	30.48705
1552.416	30.44671	30.8618	30.22869	29.72496	41.10066	31.12781
1550.487	30.23682	30.82149	30.39453	29.69348	41.19419	30.6901
1548.559	30.43512	30.87533	30.58004	29.60407	40.94566	30.94216
1546.63	30.83903	31.38426	31.19831	30.09465	41.87672	31.37876
1544.702	29.27354	30.25473	30.43809	29.29408	40.14163	29.51105
1542.774	28.83421	29.86049	30.45568	28.96619	39.31282	29.17499

1540.845	27.866	28.6741	29.81308	28.45614	38.03988	27.59211
1538.917	28.77162	29.10743	29.99504	28.49556	37.70577	28.90093
1536.988	31.31084	31.66923	31.87617	30.09966	40.89453	32.12464
1535.06	30.98612	31.82253	32.03315	30.42055	41.93512	31.41537
1533.131	29.61602	30.34172	30.81784	29.32215	39.93653	29.55832
1531.203	31.23004	31.67739	31.9018	30.07685	41.20209	31.70195
1529.274	31.28355	31.98378	32.22073	30.3684	41.99025	31.54283
1527.346	30.33125	31.24332	31.67735	29.90159	41.0018	30.35975
1525.417	30.02384	30.92002	31.4717	29.65756	40.32711	30.20948
1523.489	30.14101	31.12501	31.72222	29.86167	40.63051	30.44906
1521.56	28.747	29.84822	30.65145	29.00312	38.99328	28.54735
1519.632	30.19874	30.89541	31.43257	29.60982	39.67803	30.36372
1517.703	30.35911	31.27755	31.83984	30.15506	40.70932	30.27779
1515.775	30.07274	30.90714	31.42882	29.72257	39.88504	29.84222
1513.847	31.37181	31.99704	32.33884	30.52588	41.56398	31.19983
1511.918	31.00059	31.83075	32.1578	30.37128	41.53871	30.63616
1509.99	30.16344	31.25234	31.80875	29.95612	40.63749	29.84573
1508.061	27.89542	29.45045	30.58128	28.80783	38.55754	27.3448
1506.133	27.84575	28.68104	29.98499	28.23312	36.7541	27.06544
1504.204	31.86353	32.14666	32.606	30.56572	41.14847	31.70275
1502.276	32.29372	32.87943	33.20681	31.08162	42.7234	32.0273
1500.347	31.48798	32.35866	32.69038	30.65312	42.11906	30.90426
1498.419	30.95588	31.78313	32.29576	30.31416	41.26525	30.11705
1496.49	30.87515	31.49502	32.17456	30.13311	40.82848	30.03894
1494.562	32.05359	32.44085	32.86259	30.6946	41.94672	31.58386
1492.633	32.23632	32.83764	33.22271	31.02963	42.88491	31.65881
1490.705	30.4326	31.37339	32.11501	30.08799	41.20147	29.48048
1488.776	29.99919	30.80349	31.7805	29.67927	39.93408	29.39322
1486.848	31.44878	31.98192	32.61878	30.47251	41.33901	30.92185
1484.92	32.31674	32.67943	32.98889	30.89005	42.44774	31.67013
1482.991	32.30812	32.73262	33.0123	30.92824	42.83463	31.53744
1481.063	31.80459	32.30131	32.64677	30.51846	42.24585	30.92342
1479.134	32.09938	32.59871	32.8652	30.74017	42.58792	31.22776
1477.206	31.39857	32.10487	32.53814	30.47864	42.14264	30.22828
1475.277	30.63782	31.33824	31.96886	29.96287	41.08336	29.3569
1473.349	29.84615	30.64272	31.39686	29.61471	40.21744	28.36537
1471.42	30.46664	30.80251	31.27758	29.62657	39.87703	28.98775
1469.492	32.17626	32.38378	32.31675	30.7142	42.11776	30.8449
1467.563	31.83295	32.27464	32.09681	30.66546	42.41454	30.28254
1465.635	30.80742	31.39138	31.41823	30.03665	41.44936	29.01999
1463.706	30.99727	31.31254	31.33582	29.89125	41.08908	29.10139
1461.778	31.80635	32.10663	32.03166	30.5044	42.28366	29.97705
1459.849	30.6145	31.30729	31.57904	30.01127	41.53302	28.55204
1457.921	28.44565	29.43016	30.29882	28.72806	39.04489	26.16428
1455.993	28.9676	29.49978	30.25163	28.67753	38.21021	26.93829
1454.064	31.18892	31.56065	31.7686	30.09745	40.99056	29.30091

1452.136	31.17944	31.68445	31.76938	30.10417	41.58583	28.88817
1450.207	31.05961	31.51187	31.61385	29.96148	41.55545	28.65851
1448.279	30.42394	30.95426	31.13121	29.59988	40.97676	27.83157
1446.35	30.633	31.02928	31.126	29.58906	40.86353	28.09201
1444.422	31.00628	31.31461	31.31064	29.8876	41.49387	28.37649
1442.493	30.81622	31.13439	31.11431	29.74042	41.47023	28.0941
1440.565	30.65589	30.99994	30.98049	29.5561	41.3633	27.8065
1438.636	30.02342	30.59182	30.65136	29.19989	41.00418	27.17237
1436.708	28.60599	29.28497	29.69704	28.30952	39.35169	25.70854
1434.779	29.56103	29.84357	30.02113	28.67517	39.71296	26.95804
1432.851	29.82669	30.26158	30.41003	29.03531	40.58627	27.14326
1430.922	29.08789	29.65495	30.00342	28.51025	39.93477	26.35106
1428.994	28.85209	29.33912	29.6696	28.24223	39.50586	26.02506
1427.066	29.05581	29.44187	29.63268	28.28391	39.82013	26.26124
1425.137	28.28365	28.81231	29.04218	27.81619	39.4441	25.41404
1423.209	27.49262	28.0901	28.22318	27.17313	38.40218	24.75309
1421.28	27.25619	27.99809	28.00261	27.12363	38.56406	24.50625
1419.352	25.64682	26.5364	26.65726	26.02925	36.69324	22.76242
1417.423	25.41316	26.01883	26.08689	25.61228	35.82182	22.71033
1415.495	25.57657	26.18013	26.00922	25.59346	36.5555	22.882
1413.566	24.9148	25.61368	25.25352	25.00977	36.3751	22.07125
1411.638	23.88282	24.63546	24.19624	24.12642	35.40285	20.9616
1409.709	23.00712	23.70676	23.29339	23.28965	34.42245	20.06531
1407.781	22.16743	22.95739	22.55318	22.58478	33.66611	19.28321
1405.852	20.98856	21.92986	21.58349	21.72816	32.49948	18.08975
1403.924	20.2355	21.11222	20.75931	21.05774	31.5359	17.28481
1401.995	20.14581	20.99705	20.53679	20.86637	31.54424	17.17358
1400.067	19.51626	20.60568	20.17126	20.48424	30.9174	16.50575
1398.139	19.39937	20.51696	20.16994	20.40824	30.43691	16.45199
1396.21	19.93197	21.07149	20.84647	20.97729	31.08901	16.89722
1394.282	20.77023	21.83612	21.58419	21.6449	31.72814	17.31337
1392.353	22.56644	23.3745	23.02961	22.8948	33.61345	18.59857
1390.425	23.50057	24.30724	24.06936	23.75701	34.86397	18.97816
1388.496	23.84567	24.8252	24.67535	24.22598	35.15941	18.48104
1386.568	24.45661	25.50649	25.22216	24.72692	35.40252	17.07373
1384.639	25.91417	26.80256	26.29944	25.72688	36.90544	16.46305
1382.711	26.6647	27.54171	27.03043	26.34489	37.79372	17.37608
1380.782	27.2713	28.20672	27.72983	26.94651	38.34369	19.77436
1378.854	27.74539	28.68933	28.28849	27.44868	38.70863	21.38615
1376.925	28.2638	29.16487	28.84058	27.89063	39.10289	22.41198
1374.997	28.18327	29.16352	29.0282	27.98212	38.85648	22.55354
1373.068	28.74109	29.64176	29.44845	28.29826	39.06881	23.40149
1371.14	29.77909	30.61022	30.32275	29.02033	40.34762	24.48349
1369.212	29.89249	30.70069	30.57434	29.20521	40.5558	24.69004
1367.283	30.22605	31.00695	31.00358	29.47571	40.78873	25.14086
1365.355	30.56776	31.38319	31.41923	29.79904	41.19412	25.51396

1363.426	29.94107	30.97623	31.23828	29.5544	40.47989	24.9797
1361.498	30.22438	31.15646	31.47952	29.69085	40.32824	25.35273
1359.569	31.23571	32.0477	32.17786	30.3117	41.52293	26.31292
1357.641	31.54432	32.35208	32.36039	30.47379	41.91967	26.49806
1355.712	31.62276	32.39174	32.3313	30.49532	41.95408	26.39721
1353.784	31.7316	32.55461	32.32973	30.65019	42.05339	26.57251
1351.855	32.00108	32.75875	32.52742	30.91922	42.34336	26.95202
1349.927	32.04105	32.76941	32.56926	30.94788	42.34697	27.11564
1347.998	32.18236	32.84071	32.59401	31.02404	42.43036	27.31525
1346.07	32.30081	33.00418	32.6104	31.13271	42.58231	27.55937
1344.141	32.3646	33.10022	32.66561	31.17484	42.67215	27.73293
1342.213	32.14079	32.92627	32.54735	31.07751	42.46463	27.69065
1340.285	31.21384	32.16784	32.10961	30.58906	41.4225	26.99494
1338.356	31.20718	32.04278	32.2169	30.5529	41.106	27.20594
1336.428	32.04898	32.66173	32.80188	31.0059	42.06997	27.94298
1334.499	32.46015	32.97423	33.08346	31.12744	42.5494	28.29156
1332.571	32.51923	33.07414	33.24195	31.18647	42.70564	28.34953
1330.642	32.43777	33.07361	33.29042	31.13879	42.61769	28.32644
1328.714	32.45287	33.11218	33.46939	31.19087	42.6561	28.39982
1326.785	32.44011	33.08807	33.53202	31.20711	42.58879	28.43577
1324.857	32.44154	33.05613	33.54059	31.26477	42.63037	28.50985
1322.928	32.43518	33.00521	33.49709	31.22594	42.56293	28.59333
1321	32.37306	32.97827	33.48904	31.17962	42.44587	28.46663
1319.071	32.04214	32.76533	33.3128	31.00288	42.07286	28.24054
1317.143	32.04855	32.77447	33.3763	30.96853	42.06958	28.33598
1315.214	32.12472	32.72096	33.40617	30.91191	42.17107	28.3612
1313.286	32.08259	32.73111	33.49929	30.8898	42.20807	28.32412
1311.358	32.49693	33.12133	33.82533	31.20071	42.5756	28.70487
1309.429	32.9827	33.50591	34.19748	31.61306	43.0604	29.05577
1307.501	33.1681	33.69154	34.3867	31.77361	43.19342	29.17695
1305.572	33.42922	33.98882	34.57475	31.94937	43.39154	29.4154
1303.644	33.60194	34.13618	34.7459	32.06622	43.52168	29.57652
1301.715	33.7655	34.23994	34.90485	32.20878	43.66805	29.68314
1299.787	33.87416	34.28958	34.99692	32.22922	43.66249	29.69291
1297.858	33.97514	34.36042	34.96091	32.27999	43.76829	29.66409
1295.93	34.02257	34.41307	34.92406	32.39059	43.80029	29.68475
1294.001	34.13364	34.60465	35.08872	32.56929	43.90036	29.80279
1292.073	34.01952	34.66529	35.13943	32.52101	43.83158	29.84255
1290.144	34.02518	34.60322	35.20865	32.53392	43.84485	29.82739
1288.216	34.05719	34.53846	35.17229	32.50873	43.78587	29.76794
1286.287	34.20904	34.65038	35.22821	32.58915	43.84439	29.90599
1284.359	34.24021	34.77505	35.29905	32.58714	43.91233	30.02013
1282.431	34.27645	34.85695	35.30582	32.62473	43.93939	30.03804
1280.502	34.26383	34.91584	35.26653	32.64814	43.92395	30.00794
1278.574	34.33666	34.93784	35.33366	32.67326	43.99847	30.00081
1276.645	34.36586	34.92805	35.28959	32.72353	43.98677	30.0149

1274.717	34.4171	34.99806	35.3088	32.81697	44.00973	30.03167
1272.788	34.38698	34.98636	35.29865	32.77277	43.95047	29.99526
1270.86	34.38364	34.92096	35.3019	32.73206	43.88502	29.98847
1268.931	34.46412	34.97159	35.36132	32.73173	43.91307	30.00187
1267.003	34.50085	34.95908	35.33746	32.78662	43.95852	30.0241
1265.074	34.50282	34.91525	35.28634	32.77253	43.93514	29.99757
1263.146	34.59536	35.01092	35.34097	32.81136	44.02656	30.04042
1261.217	34.53184	35.07129	35.37877	32.78395	44.03839	29.98703
1259.289	34.59856	35.09261	35.43251	32.80577	44.01471	30.0173
1257.36	34.69761	35.16486	35.47849	32.94285	44.10266	30.10574
1255.432	34.73882	35.20406	35.51362	33.05518	44.23648	30.10832
1253.504	34.78143	35.2474	35.5556	33.10861	44.252	30.08467
1251.575	34.80109	35.29533	35.62222	33.12246	44.275	30.02776
1249.647	34.83193	35.36971	35.64095	33.09034	44.23337	29.98741
1247.718	34.88036	35.43885	35.65045	33.09581	44.2501	30.04925
1245.79	34.88477	35.45523	35.57895	33.09634	44.24596	30.05275
1243.861	34.9229	35.49085	35.60279	33.15357	44.21756	30.02563
1241.933	34.95657	35.51732	35.65319	33.19501	44.25056	30.0955
1240.004	34.94852	35.57713	35.7404	33.16024	44.32255	30.15045
1238.076	34.9873	35.61189	35.79126	33.16422	44.33299	30.18909
1236.147	35.05969	35.61819	35.89951	33.23999	44.42717	30.29888
1234.219	35.0629	35.59671	35.94211	33.27792	44.44545	30.35081
1232.29	35.07609	35.66516	36.01731	33.32672	44.45208	30.35841
1230.362	35.06371	35.74783	36.07534	33.33308	44.48082	30.41772
1228.433	35.07268	35.76797	36.15526	33.36363	44.47887	30.46101
1226.505	35.09191	35.74628	36.18841	33.32695	44.37623	30.4449
1224.577	35.11543	35.77555	36.20497	33.35762	44.43322	30.47495
1222.648	35.1694	35.78004	36.21776	33.39751	44.49073	30.56485
1220.72	35.20479	35.74942	36.22132	33.41066	44.50747	30.5732
1218.791	35.17679	35.73005	36.19224	33.42413	44.51093	30.52396
1216.863	35.23971	35.72277	36.20449	33.49279	44.55686	30.59684
1214.934	35.26524	35.75697	36.20783	33.4788	44.53205	30.6726
1213.006	35.2062	35.77473	36.12755	33.51258	44.5307	30.6546
1211.077	35.19658	35.78684	36.12056	33.51247	44.50355	30.62626
1209.149	35.27487	35.82366	36.24595	33.59742	44.59673	30.67625
1207.22	35.35299	35.8774	36.30162	33.57423	44.61805	30.68806
1205.292	35.41395	35.97588	36.34207	33.55754	44.6592	30.73044
1203.363	35.40687	36.04933	36.44216	33.60529	44.67751	30.81041
1201.435	35.48752	36.07132	36.51859	33.6992	44.77604	30.88084
1199.506	35.54541	36.04435	36.52448	33.73043	44.74754	30.89782
1197.578	35.59628	36.12634	36.53736	33.78992	44.77597	30.89823
1195.65	35.64036	36.20741	36.50027	33.80398	44.81942	30.94231
1193.721	35.63878	36.26852	36.56935	33.85331	44.82941	30.96525
1191.793	35.66316	36.29377	36.61792	33.85279	44.83545	30.98545
1189.864	35.7307	36.30885	36.67146	33.8806	44.89435	31.04725
1187.936	35.69138	36.33157	36.59659	33.88634	44.84326	31.03438

1186.007	35.71276	36.3644	36.6248	33.93783	44.90335	31.04943
1184.079	35.70736	36.38827	36.71626	33.91788	44.90595	31.04356
1182.15	35.72134	36.39195	36.75758	33.92424	44.92156	30.95049
1180.222	35.72591	36.37598	36.71225	33.86932	44.89818	30.8734
1178.293	35.71219	36.38595	36.72558	33.95185	44.96836	30.89311
1176.365	35.72	36.39471	36.73642	33.96622	44.94871	30.85075
1174.436	35.75293	36.3986	36.74163	33.94464	44.89557	30.75529
1172.508	35.79994	36.3422	36.73697	33.94741	44.90168	30.81384
1170.579	35.89272	36.37848	36.7828	34.02435	45.02839	30.9181
1168.651	35.85581	36.34978	36.72577	34.01819	44.97595	30.89315
1166.723	35.84202	36.40002	36.73445	34.02672	44.95168	30.91554
1164.794	35.82333	36.40899	36.80044	34.04573	44.89888	30.93543
1162.866	35.87609	36.42923	36.7975	34.02511	44.97322	30.96595
1160.937	35.86775	36.47317	36.73034	34.00904	45.03541	30.97961
1159.009	35.85574	36.48905	36.73235	33.9905	45.11169	31.04528
1157.08	35.83889	36.39522	36.72589	33.95322	44.97898	31.08476
1155.152	35.87589	36.36202	36.79256	33.9764	44.95988	31.02955
1153.223	35.86013	36.38905	36.85423	33.9498	45.00399	30.95948
1151.295	35.83499	36.43103	36.79763	33.96892	44.96241	30.91841
1149.366	35.84267	36.41	36.73715	33.98069	44.84212	30.79622
1147.438	35.87341	36.37702	36.72692	34.00261	44.92901	30.77124
1145.509	35.78891	36.36677	36.64134	33.99201	44.948	30.71494
1143.581	35.72892	36.42789	36.63331	33.92865	44.92578	30.66018
1141.652	35.66914	36.4122	36.6334	33.84846	44.881	30.53217
1139.724	35.68596	36.29964	36.63933	33.86943	44.91433	30.38188
1137.796	35.69216	36.13511	36.55661	33.80387	44.79712	30.2203
1135.867	35.55993	36.05014	36.59202	33.69324	44.7551	30.13244
1133.939	35.4467	36.03669	36.47241	33.63697	44.68338	30.06789
1132.01	35.40406	35.97493	36.29623	33.56108	44.63365	29.97265
1130.082	35.33996	35.89687	36.23824	33.48278	44.5243	29.84227
1128.153	35.33646	35.93705	36.24182	33.53948	44.51099	29.82795
1126.225	35.18333	35.92746	36.15905	33.50114	44.4393	29.72871
1124.296	35.10757	35.96008	36.04263	33.56401	44.4641	29.547
1122.368	35.09021	35.88889	35.90107	33.52241	44.4091	29.47322
1120.439	35.0414	35.78392	35.74445	33.40653	44.34271	29.51015
1118.511	34.91906	35.7616	35.55287	33.31309	44.28216	29.43854
1116.582	34.96073	35.84661	35.35941	33.32883	44.33922	29.37341
1114.654	34.97332	35.76478	34.98736	33.36729	44.29612	29.26264
1112.725	34.93694	35.70556	34.65346	33.3219	44.16805	29.2765
1110.797	34.9496	35.78889	34.61076	33.30475	44.14949	29.38153
1108.869	35.08278	35.91503	34.82329	33.37798	44.26598	29.45741
1106.94	35.1854	35.98308	35.14048	33.44976	44.41854	29.40109
1105.012	35.38722	36.18046	35.5516	33.66232	44.59118	29.37894
1103.083	35.5313	36.26728	35.8893	33.69356	44.56576	29.44082
1101.155	35.68789	36.44585	36.1288	33.75143	44.75756	29.51325
1099.226	35.81129	36.57616	36.29807	33.83666	44.78297	29.52926

1097.298	35.95393	36.66801	36.47229	33.94487	44.88466	29.4859
1095.369	35.97694	36.78167	36.5269	33.92144	44.9873	29.42876
1093.441	36.00673	36.8278	36.61912	34.01769	45.12856	29.54029
1091.512	36.11322	36.86587	36.68382	34.03201	45.1676	29.60729
1089.584	36.301	36.91436	36.76615	34.08436	45.2206	29.66534
1087.655	36.3144	36.97676	36.8913	34.09657	45.25989	29.72953
1085.727	36.30644	37.03128	36.96444	34.17798	45.31224	29.68075
1083.798	36.25249	36.9941	36.96622	34.24231	45.26312	29.62249
1081.87	36.26703	37.01382	36.94629	34.25629	45.21543	29.62394
1079.942	36.23413	36.90042	36.94607	34.13383	45.18937	29.48554
1078.013	36.13595	36.80342	36.96484	34.04792	45.22484	29.40628
1076.085	35.99818	36.70018	36.87474	34.07553	45.14325	29.52983
1074.156	35.90523	36.69472	36.80108	34.10948	45.11481	29.57516
1072.228	35.71217	36.56611	36.61929	33.98824	44.8993	29.47856
1070.299	35.63652	36.48817	36.25956	33.88747	44.80321	29.43111
1068.371	35.5831	36.40175	35.87075	33.74667	44.74535	29.55787
1066.442	35.51709	36.36641	35.84393	33.80072	44.7972	29.67509
1064.514	35.58435	36.46429	36.1006	33.97358	44.81913	29.72805
1062.585	35.72468	36.66434	36.5184	34.00507	44.90889	29.79954
1060.657	35.86984	36.79352	36.85094	33.95859	44.96448	29.87715
1058.728	36.0982	36.91045	37.18489	34.08885	45.04399	29.96795
1056.8	36.18606	37.13287	37.43622	34.31764	45.10812	30.13142
1054.871	36.42202	37.3215	37.63661	34.47146	45.29026	30.20815
1052.943	36.56628	37.37499	37.75146	34.48074	45.35489	30.26529
1051.015	36.68161	37.44884	37.97026	34.55516	45.46559	30.4194
1049.086	36.71125	37.50863	38.09634	34.61049	45.49495	30.48187
1047.158	36.84773	37.57101	38.15396	34.71255	45.57685	30.48805
1045.229	36.95189	37.65115	38.21614	34.65926	45.57738	30.45384
1043.301	36.98222	37.73349	38.26006	34.69465	45.6725	30.50612
1041.372	37.01307	37.87626	38.31046	34.75974	45.78223	30.60487
1039.444	37.12196	37.9985	38.38395	34.82842	45.85398	30.68388
1037.515	37.1228	38.00129	38.44459	34.83747	45.80161	30.6524
1035.587	37.09858	37.97018	38.46213	34.92706	45.86649	30.65348
1033.658	37.19002	37.97669	38.36593	34.94734	45.85115	30.71654
1031.73	37.25885	38.05085	38.54622	34.97964	45.83	30.74854
1029.801	37.26634	38.07078	38.66782	34.95927	45.87549	30.65828
1027.873	37.36453	38.07932	38.64557	35.01559	45.94984	30.6768
1025.944	37.48725	38.15852	38.79954	35.06784	45.95475	30.83071
1024.016	37.54945	38.32196	39.04707	35.17384	45.92444	30.90752
1022.087	37.50602	38.40993	38.99516	35.15677	45.89637	30.89287
1020.159	37.48315	38.34861	38.85505	35.14932	46.02805	30.89944
1018.231	37.47254	38.26185	38.90541	35.28321	46.10937	30.98152
1016.302	37.58932	38.41647	39.08558	35.38916	46.25305	31.07115
1014.374	37.62292	38.60486	39.23194	35.44907	46.32492	31.26246
1012.445	37.65492	38.69167	39.33733	35.52233	46.38638	31.41612
1010.517	37.76877	38.67418	39.31314	35.56594	46.37415	31.49525

1008.588	37.8834	38.70086	39.37812	35.57391	46.44246	31.60123
1006.66	37.89345	38.75486	39.45259	35.60572	46.43544	31.71675
1004.731	37.95324	38.81081	39.47807	35.61985	46.59719	31.80502
1002.803	37.95061	38.85613	39.51461	35.5899	46.58002	31.81259
1000.874	37.97597	38.8773	39.61507	35.7359	46.62173	31.9692
998.9459	37.9806	38.84101	39.57407	35.85315	46.61645	32.12924
997.0174	38.09101	38.98972	39.52359	35.97834	46.71413	32.27082
995.0889	38.07111	39.03347	39.43404	35.94338	46.74252	32.35474
993.1605	38.08528	39.11424	39.57312	36.0213	46.78641	32.30699
991.232	38.13184	39.10632	39.77537	36.04308	46.74534	32.33549
989.3035	38.24028	39.27775	39.98545	36.11206	46.79474	32.5219
987.3751	38.34121	39.39169	40.1674	36.1535	46.95246	32.64764
985.4466	38.45065	39.38905	40.28693	36.2229	47.17793	32.81948
983.5181	38.51028	39.3986	40.23473	36.2976	47.13404	32.93979
981.5897	38.60621	39.41066	40.35907	36.43602	47.19564	33.01217
979.6612	38.67127	39.50985	40.4267	36.48009	47.3059	33.0654
977.7327	38.75438	39.71067	40.58378	36.4863	47.37249	33.11943
975.8043	38.71721	39.80252	40.63482	36.5387	47.2583	33.15541
973.8758	38.70749	39.82357	40.659	36.623	47.32945	33.22026
971.9473	38.77866	39.83948	40.72248	36.66226	47.35234	33.3211
970.0189	38.86266	39.94077	40.78247	36.79626	47.51802	33.42931
968.0904	38.90426	40.09766	40.82993	36.8222	47.57483	33.59412
966.1619	39.05902	40.11709	40.92632	36.8865	47.58244	33.65661
964.2335	39.14098	40.1869	41.02018	36.99168	47.66611	33.74087
962.305	39.22379	40.24902	41.13271	37.05696	47.84025	33.85952
960.3765	39.2816	40.26511	41.22734	37.11443	47.79869	33.81608
958.4481	39.31363	40.2922	41.17054	37.29185	47.76971	33.71293
956.5196	39.3427	40.27551	41.14156	37.23597	47.7153	33.71439
954.5911	39.36928	40.35614	41.17039	37.11893	47.7469	33.77649
952.6627	39.36607	40.38821	41.21432	37.1489	47.81205	33.85899
950.7342	39.40883	40.43039	41.28099	37.29336	47.89946	33.90752
948.8057	39.4535	40.56344	41.30776	37.36073	47.90296	33.83477
946.8773	39.48539	40.67394	41.37611	37.36901	48.03568	33.92001
944.9488	39.44023	40.72332	41.48756	37.42241	48.01455	34.01212
943.0203	39.53146	40.76019	41.57158	37.42419	48.022	34.07941
941.0919	39.53472	40.66616	41.56786	37.35886	47.95406	34.21786
939.1634	39.65474	40.79425	41.69188	37.55447	48.09759	34.3919
937.2349	39.72319	40.86956	41.74929	37.56159	48.22109	34.39442
935.3065	39.74839	40.84937	41.73936	37.58945	48.23429	34.43331
933.378	39.72242	40.83905	41.69336	37.60714	48.16749	34.42878
931.4495	39.72154	40.9168	41.77903	37.67414	48.19174	34.55111
929.5211	39.69342	41.00012	41.80812	37.74488	48.25687	34.62985
927.5926	39.72903	41.02062	41.70121	37.69575	48.29019	34.69927
925.6641	39.71317	40.98813	41.50933	37.64703	48.17278	34.74828
923.7357	39.75343	40.94224	41.33513	37.69578	48.14828	34.75877
921.8072	39.68373	40.9348	41.21858	37.69297	48.06647	34.74506

919.8787	39.81793	41.06676	41.44092	37.74798	48.17702	34.858
917.9503	39.90284	41.04797	41.68719	37.75098	48.24396	34.92106
916.0218	39.86897	41.09912	41.9808	37.86644	48.36527	35.01593
914.0933	39.8704	41.1429	42.16468	37.89776	48.37283	35.0297
912.1649	39.97618	41.17773	42.29214	37.9092	48.45068	35.1411
910.2364	40.00729	41.26734	42.2749	38.00689	48.49698	35.15268
908.3079	40.07876	41.30112	42.28833	38.16352	48.57332	35.09423
906.3795	40.07814	41.28289	42.26561	38.15121	48.54441	35.05922
904.451	40.01509	41.27904	42.41072	38.16867	48.64023	35.2098
902.5225	40.10153	41.40415	42.54707	38.17058	48.62349	35.36291
900.5941	40.21618	41.50323	42.56008	38.22809	48.56899	35.42651
898.6656	40.23222	41.47913	42.54757	38.22252	48.45667	35.44796
896.7371	40.34212	41.49767	42.58867	38.29279	48.58455	35.46825
894.8087	40.40036	41.53962	42.5986	38.32421	48.65296	35.57461
892.8802	40.34777	41.5784	42.66925	38.34955	48.6905	35.59204
890.9517	40.36529	41.58919	42.65264	38.37652	48.73486	35.50092
889.0233	40.37177	41.72337	42.64138	38.32595	48.82122	35.63615
887.0948	40.32511	41.7948	42.54402	38.23906	48.88944	35.72789
885.1663	40.30282	41.93948	42.63432	38.43353	48.85546	35.83855
883.2379	40.32942	41.70959	42.66119	38.57022	48.70904	35.84303
881.3094	40.59264	41.62741	42.83955	38.59039	48.88128	35.88477
879.3809	40.56135	41.77713	42.91352	38.49605	48.98971	35.82282
877.4525	40.48905	41.80227	42.9036	38.54637	48.94357	35.82986
875.524	40.32986	41.77251	42.88803	38.52568	48.90833	35.90123
873.5955	40.3162	41.77706	42.90731	38.53172	48.96289	35.87487
871.6671	40.44211	41.75323	42.98696	38.58372	48.97199	35.79212
869.7386	40.60869	41.87966	43.11513	38.69062	49.05542	35.80379
867.8101	40.69384	42.05629	43.10889	38.81739	49.07862	35.90191
865.8817	40.66747	42.13986	43.13861	38.85174	49.17888	36.12255
863.9532	40.55297	42.00433	43.16034	38.74678	49.09948	36.05747
862.0247	40.65443	41.94062	43.34143	38.85353	49.0776	35.98192
860.0963	40.6982	42.00755	43.27352	38.81403	48.93579	35.85181
858.1678	40.68712	42.06868	43.12242	38.73952	48.99759	35.83817
856.2393	40.54433	41.98001	43.19548	38.68731	49.00721	35.87578
854.3109	40.5927	41.85891	43.24659	38.73059	48.9666	35.79289
852.3824	40.531	41.77573	43.16135	38.66382	48.84884	35.73658
850.4539	40.60644	41.96362	43.19893	38.78791	48.95803	35.93405
848.5255	40.51914	41.90041	43.20718	38.73837	48.93567	35.90242
846.597	40.47778	41.82896	43.34754	38.74142	48.94083	35.7556
844.6685	40.42683	41.76457	43.24224	38.68815	49.02471	35.67295
842.7401	40.44231	41.79098	43.12805	38.77386	48.99953	35.65698
840.8116	40.43221	41.73856	43.19862	38.83432	48.89395	35.48584
838.8831	40.58109	41.90822	43.28548	38.81961	48.96693	35.34016
836.9547	40.58147	41.96708	43.29564	38.79936	48.93135	35.44886
835.0262	40.54784	42.0065	43.43552	38.88266	48.91529	35.49564
833.0977	40.56444	41.91345	43.34644	38.81639	48.96204	35.43301

831.1693	40.61703	41.90074	43.35932	38.85735	49.02301	35.51421
829.2408	40.5174	41.94625	43.40142	38.79197	48.8676	35.40699
827.3123	40.55556	41.96734	43.42559	38.83281	48.9775	35.20658
825.3839	40.61139	41.88268	43.42686	38.83275	49.10797	35.17539
823.4554	40.69485	41.93235	43.57801	38.92015	49.06257	35.25617
821.5269	40.56598	42.09863	43.69853	38.94836	48.91518	35.36977
819.5985	40.4925	42.1097	43.60497	38.95181	48.99017	35.63354
817.67	40.54479	41.98581	43.46243	38.93843	49.03193	35.86425
815.7415	40.51318	41.9809	43.50388	38.93774	49.02394	35.96674
813.8131	40.40204	42.03223	43.61004	38.8022	49.03083	35.96851
811.8846	40.44	42.21772	43.58265	38.76723	49.07133	35.95846
809.9561	40.43458	42.14041	43.50181	38.79496	49.00896	35.97451
808.0277	40.48513	42.16681	43.49468	38.90085	49.01521	36.00806
806.0992	40.428	42.17397	43.46246	38.91163	48.81023	36.01246
804.1707	40.37522	42.13293	43.60225	39.00502	48.85896	36.0799
802.2423	40.39574	42.30119	43.59933	39.00906	48.99482	36.06585
800.3138	40.5473	42.17975	43.71247	39.00252	49.15292	36.20182
798.3853	40.4786	42.05074	43.67957	38.8817	49.11364	36.30217
796.4569	40.53252	42.0118	43.64356	38.96185	49.1126	36.20516
794.5284	40.45643	41.97807	43.66245	38.95031	49.0307	36.19349
792.5999	40.51253	41.92012	43.78361	38.9864	49.12608	36.29076
790.6715	40.41395	41.74656	43.72739	38.93103	48.93047	36.20621
788.743	40.42386	41.73015	43.62191	38.97785	49.11196	36.36988
786.8145	40.3594	41.79959	43.44813	38.89141	49.18191	36.31325
784.8861	40.24201	41.72989	43.4591	38.88712	49.00571	36.19238
782.9576	40.07808	41.42461	43.30112	38.81985	48.65904	36.08463
781.0291	39.86378	41.2585	42.92829	38.69196	48.56055	35.87157
779.1007	39.75933	41.25413	42.9163	38.61483	48.54878	35.85452
777.1722	39.98255	41.46349	43.27721	38.91328	48.77749	36.03217
775.2437	40.13027	41.71119	43.40768	39.03178	48.91679	36.19593
773.3153	40.25589	41.81244	43.5607	39.09858	49.07965	36.325
771.3868	40.27658	41.98018	43.70017	39.24244	49.09459	36.42067
769.4583	40.37197	42.22858	43.90171	39.40286	49.18173	36.52362
767.5299	40.40187	42.31953	43.88715	39.29492	49.23755	36.5264
765.6014	40.59028	42.27415	43.76534	39.31121	49.20798	36.47812
763.6729	40.54032	42.1256	43.77216	39.27404	49.20775	36.43026
761.7445	40.43237	42.16885	44.07551	39.4296	49.30778	36.52806
759.816	40.45289	42.28861	44.19101	39.48727	49.23835	36.58525
757.8875	40.45078	42.32099	44.14462	39.4303	49.22847	36.47137
755.9591	40.42037	42.1824	44.01934	39.34321	49.245	36.36519
754.0306	40.57029	42.25807	44.05942	39.50558	49.45309	36.54254
752.1021	40.61381	42.4057	44.09924	39.74459	49.31582	36.71912
750.1737	40.55027	42.44534	44.20325	39.71571	49.36938	36.74245
748.2452	40.36591	42.20444	44.27558	39.45179	49.35972	36.6805
746.3167	40.31913	42.17349	44.25354	39.39319	49.36642	36.80946
744.3882	40.38345	42.34167	44.25179	39.43147	49.41683	36.86615

742.4598	40.54696	42.36353	44.25893	39.46426	49.48309	36.65192
740.5313	40.50916	42.22135	44.26445	39.47691	49.43927	36.67122
738.6028	40.41217	42.23988	44.213	39.52805	49.38248	36.73389
736.6744	40.28999	42.1129	43.98494	39.52291	49.20504	36.60144
734.7459	40.26508	42.20468	44.01665	39.53292	49.20094	36.57654
732.8174	40.28075	42.2599	44.07118	39.15222	49.1674	36.63503
730.889	40.38437	42.13409	43.99923	39.11893	49.14008	36.76694
728.9605	40.31364	42.05555	43.77411	39.18734	49.18014	36.6669
727.032	40.17629	42.119	43.89426	39.2411	49.46097	36.50933
725.1036	40.05074	42.05537	43.81205	39.12904	49.25066	36.41957
723.1751	39.96683	41.99942	43.65438	39.2165	49.20491	36.48584
721.2466	39.91357	41.94427	43.44642	39.09616	49.11688	36.30628
719.3182	39.96989	41.98096	43.35492	39.12559	49.2242	36.23743
717.3897	39.89568	41.88951	43.48968	39.07748	49.18638	36.10778
715.4612	39.97392	41.81688	43.58137	39.12298	49.05595	35.95265
713.5328	39.91966	41.9818	43.63353	39.19366	48.95827	35.87938
711.6043	39.84244	41.88382	43.63181	39.17535	49.04507	35.9596
709.6758	39.81161	41.84179	43.69425	38.92886	48.98935	36.17204
707.7474	39.71484	41.72684	43.72438	38.89714	48.89018	36.22425
705.8189	39.60236	41.78213	43.70277	38.97137	48.75698	36.03317
703.8904	39.63559	41.8576	43.80316	39.09766	48.85677	35.93837
701.962	39.5944	41.68071	43.85847	38.95586	48.79646	35.90263
700.0335	39.76019	41.4884	43.61489	38.89779	48.85727	35.90652
698.105	39.71739	41.40594	43.52382	38.82151	48.80662	35.85596
696.1766	39.6698	41.70507	43.67425	38.98619	48.97598	35.90425
694.2481	39.4856	41.67815	43.59448	39.10289	48.939	35.95102
692.3196	39.46374	41.46507	43.67491	39.02498	48.93251	35.75454
690.3912	39.45565	41.42172	43.57929	38.88592	48.93378	35.61263
688.4627	39.50102	41.54744	43.48834	38.92014	48.7805	35.79342
686.5342	39.72297	41.67022	43.62872	38.98536	48.95435	35.80199
684.6058	39.76139	41.63616	43.59904	39.02019	49.22215	35.5538
682.6773	39.85767	41.6811	43.64686	38.92625	49.25074	35.82026
680.7488	39.57015	41.76012	43.67941	38.90149	49.01524	36.07208
678.8204	39.34809	41.69268	43.37427	38.87921	48.87418	36.00775
676.8919	39.43755	41.56292	43.39859	39.0014	48.97029	35.75658
674.9634	39.5196	41.42043	43.43295	39.06236	48.90034	35.75536
673.035	39.42465	41.34428	43.12574	38.91532	48.87929	35.56308
671.1065	39.06417	41.12981	42.83969	38.61919	48.62864	35.18933
669.178	38.53497	40.34251	42.45307	38.17553	48.22062	34.14882
667.2496	40.33189	41.76001	43.99785	39.40115	49.53512	35.87075
665.3211	40.49352	42.61909	44.60422	39.74043	49.90776	36.46272
663.3926	39.68068	41.83579	43.8335	39.1	49.27078	35.79359
661.4642	39.47497	41.5711	43.48272	38.9579	49.06635	35.5748
659.5357	39.47628	41.38954	43.04897	38.86998	48.85269	35.43859
657.6072	39.6137	41.69689	42.77262	39.25349	49.139	35.66813
655.6788	39.49806	41.74363	42.64533	39.32007	49.2658	35.66908

653.7503	39.61453	41.68431	42.75259	39.30653	49.11961	35.61886
651.8218	39.49765	41.50725	42.71176	38.91393	48.84222	35.4722
649.8934	39.37536	41.46002	42.68043	38.69124	49.20682	35.44856
647.9649	39.21886	41.493	42.2642	38.72148	49.15108	35.55719
646.0364	39.33327	41.75492	42.13915	39.05795	49.28659	35.54896
644.108	39.34927	41.80406	41.78977	38.99645	49.19941	35.28904
642.1795	39.13363	41.74214	41.51359	39.11588	48.96849	35.32155
640.251	38.80707	41.47667	41.40009	39.1715	48.94234	35.43889
638.3226	38.87266	41.34937	41.42711	38.91736	49.38215	35.53227
636.3941	38.69507	41.34504	41.1601	38.59011	49.03325	35.2728
634.4656	38.69378	41.15739	41.12879	38.79446	48.71698	35.14306
632.5372	38.94119	41.29451	41.00018	38.7475	48.86463	35.30351
630.6087	39.11248	41.47542	40.98839	38.66472	49.46926	35.33365
628.6802	38.84682	41.61346	41.09925	38.57969	49.5354	35.211
626.7518	38.68551	41.61319	41.21058	38.85137	49.32833	35.18517
624.8233	38.58635	41.33861	40.9958	39.04127	49.03453	35.35278
622.8948	38.95922	41.51454	41.00754	39.04917	49.59322	35.93351
620.9664	38.97573	41.56998	40.88528	38.9163	49.37264	35.68402
619.0379	38.83787	41.61748	40.97657	38.97579	49.3027	35.56893
617.1094	38.40838	41.90114	41.2622	38.99052	49.18062	35.6807
615.181	38.31413	42.79847	41.33807	38.70164	49.7705	36.03859
613.2525	40.27636	42.92301	41.18768	39.42428	50.35811	35.78764
611.324	40.40492	41.30577	41.57446	39.85805	50.0213	34.65151
609.3956	39.54556	40.69329	41.33707	39.36145	49.69509	35.06141
607.4671	39.87623	41.45366	41.34218	39.64956	49.86633	36.0204
605.5386	39.80571	42.01309	41.78495	39.80565	49.70754	35.99791
603.6102	39.78822	42.0963	42.41896	40.01929	49.80236	36.18423
601.6817	39.92028	42.38454	42.54775	39.87788	49.8905	36.04761
599.7532	39.88086	42.47838	42.67227	40.02995	49.93117	36.14423
597.8248	40.11802	42.49634	42.88474	40.16709	50.3909	36.41241
595.8963	40.38415	42.98436	43.12449	40.46241	50.58766	36.23633
593.9678	40.26261	43.32044	43.38467	40.42212	50.42994	35.94282
592.0394	40.28699	42.84787	43.77715	40.60867	50.04722	35.75295
590.1109	40.66931	42.73422	43.98753	40.84169	50.54176	36.14197
588.1824	40.86309	43.22381	44.15273	40.67511	51.13114	36.883
586.254	40.89249	43.1509	44.26754	40.51813	50.77839	36.77469
584.3255	41.07451	43.38522	44.89122	40.88368	50.63254	36.82784
582.397	40.92425	43.63785	44.8372	41.18923	50.86895	36.78383
580.4686	40.84972	43.66196	44.77439	40.90543	50.90613	36.38589
578.5401	40.79526	43.05084	44.94882	40.48116	50.99554	36.31876
576.6116	40.98295	42.93953	44.84797	40.70467	50.73597	36.25182
574.6832	41.12571	43.52481	45.02202	41.02046	50.83382	36.44869
572.7547	41.17392	43.54403	45.22947	41.0675	51.08588	36.41561
570.8262	40.6838	43.38791	45.1965	40.91409	51.04383	36.40428
568.8978	40.66997	43.39892	45.23167	40.94578	50.73736	36.43747
566.9693	41.15114	43.52196	45.16057	40.92938	50.20476	36.19702

565.0408	40.99883	43.52898	45.39876	41.07214	50.46331	35.90706
563.1124	40.88954	43.0232	45.31336	40.97318	50.2509	35.6447
561.1839	41.18241	42.90251	45.29038	40.8268	50.19275	35.80695
559.2554	40.75945	43.27731	45.37222	40.75452	50.68251	35.91061
557.327	40.52691	43.29959	45.49415	40.73529	50.65828	35.80685
555.3985	40.71844	42.82477	45.35197	40.38336	50.33537	35.62892
553.47	40.80666	42.86142	45.13367	40.27371	50.03516	35.76533
551.5416	40.31043	42.75396	44.41858	40.01256	49.62586	35.47393
549.6131	40.16486	42.30416	44.13077	39.8097	49.55787	35.09721
547.6846	39.73617	42.52224	44.13302	40.02417	49.97371	34.9473
545.7562	39.28896	42.74061	45.03171	40.90757	50.63739	34.90028
543.8276	39.70464	43.45731	45.10971	40.55087	49.60916	35.66893
541.8992	40.29781	42.92126	44.80649	40.07635	49.17775	35.50506
539.9707	39.95135	41.75361	44.17557	39.76911	48.99517	34.3427
538.0422	39.62365	41.74421	44.0901	39.73343	49.09367	34.38854
536.1138	39.35194	41.72948	43.78159	39.68118	49.01283	34.16913
534.1853	39.18104	42.27229	43.74158	39.62296	49.25	33.99306
532.2568	38.94971	42.13691	43.55076	39.46727	49.20328	34.05531
530.3284	38.74023	41.87146	43.43972	39.02517	49.13684	34.10099
528.3999	38.44016	41.35239	43.03942	38.37268	48.7862	33.54643
526.4714	38.18413	40.9817	42.93954	38.32668	47.99912	32.60648
524.543	38.0908	40.69418	43.0717	38.79858	48.03413	32.56598
522.6145	38.15576	40.72499	42.65297	38.5727	48.44196	33.12696
520.686	37.93903	40.45127	41.8498	37.73027	47.45751	33.09832
518.7576	37.79492	40.44163	42.41976	37.86601	47.19461	32.47332
516.8291	37.62682	40.47644	42.47095	38.02595	47.28785	32.5647
514.9006	38.11624	41.09507	42.5299	37.91937	47.96351	33.23008
512.9722	37.72223	40.70865	42.33013	37.69007	47.97833	33.01388
511.0437	37.68795	40.42459	42.32948	38.13816	47.8933	32.61411
509.1153	37.37397	40.31868	41.74395	37.72468	47.9534	32.36975
507.1868	37.46046	40.40796	41.36098	38.16695	48.10564	33.11449
505.2583	37.87251	40.50256	41.82874	38.0786	48.08241	33.37595
503.3299	37.64776	40.56918	41.44619	37.40064	47.83658	32.96198
501.4014	37.52278	40.67062	41.06065	37.44979	47.5376	33.49585
499.4729	38.33731	41.39087	41.30011	38.29311	48.26488	34.2794
497.5445	38.28596	41.02132	41.22172	37.85143	47.93174	34.24017
495.616	37.96493	40.64613	40.96965	37.7287	47.88523	33.42973
493.6875	37.48264	40.06089	40.32892	37.82757	47.25193	32.11489
491.7591	37.69514	40.5263	39.78157	38.19827	47.23289	32.30104
489.8306	38.63145	40.99532	39.94345	38.32348	47.71689	33.17067
487.9021	38.58805	40.87798	39.97034	38.50399	48.24916	33.91052
485.9737	37.09667	40.14813	39.70051	38.40374	47.74486	33.65253
484.0452	37.46492	40.68487	40.2232	38.82799	47.76555	33.76016
482.1167	38.27843	41.73199	40.36747	38.83682	48.13597	33.9968
480.1883	38.02523	42.175	40.02787	37.02958	47.66235	34.09708
478.2598	39.03088	43.58308	40.02618	34.91443	45.97646	34.30533

476.3313	39.97508	45.18286	40.9486	38.65503	47.93037	36.25098
474.4029	38.74496	41.86089	40.63272	39.73597	49.17396	38.82214
472.4744	38.61629	39.23945	40.95333	38.94712	48.84233	36.45929
470.5459	39.68988	41.03592	41.54862	39.83622	49.89859	35.41823
468.6175	39.36782	42.39452	41.92871	39.98889	49.21204	34.3763
466.689	39.90635	43.02444	42.64544	40.10355	49.32183	35.21308
464.7605	40.7736	43.14824	43.14141	40.91085	49.60464	35.94945
462.8321	41.24096	42.11172	43.32014	41.06716	48.89868	35.9449
460.9036	41.59545	42.27522	42.85306	40.97799	48.4769	36.13423
458.9751	40.72797	41.76821	42.58878	40.60891	47.60018	35.65109
457.0467	41.72638	43.43188	45.09728	41.84002	49.23547	35.99985
455.1182	42.24651	44.31579	45.82128	42.69807	50.97314	37.48213
453.1897	41.08492	43.59211	44.94854	42.5947	50.7767	37.33287
451.2613	42.33358	44.99192	45.19895	43.01487	51.4123	37.50835
449.3328	43.43629	45.37572	45.19947	43.26224	51.46791	37.74593
447.4043	42.39929	44.81992	44.79763	42.88913	51.19236	37.7845
445.4759	42.45635	45.03769	45.16746	42.67514	50.91341	38.61184
443.5474	43.1762	45.2993	45.60283	42.50238	50.12	39.74968
441.6189	44.32283	46.29527	46.5849	43.82806	51.1789	40.57589
439.6905	44.13072	46.59337	46.04069	44.56116	51.99684	39.96334
437.762	42.20441	46.49611	45.72689	43.42241	51.08318	37.84501
435.8335	41.93634	46.18551	45.44277	43.36387	49.96432	37.24539
433.9051	42.56862	45.84504	44.98355	44.01762	49.92591	37.98921
431.9766	42.72139	45.24833	44.59209	43.08271	49.95297	38.83901
430.0481	43.73473	44.49503	44.63977	42.89626	50.02213	38.60038
428.1197	44.05465	44.7658	45.13555	43.1478	49.96973	38.04077
426.1912	43.27458	44.17667	45.14301	42.74331	49.87806	37.09799
424.2627	41.82434	43.44731	42.90765	41.20623	49.82458	36.52236
422.3343	40.87192	42.13485	42.30828	41.55815	49.67327	37.62696
420.4058	37.83176	38.85549	42.55213	40.67276	46.89877	35.0064
418.4773	38.95089	41.56875	43.38977	41.18235	46.54306	36.89133
416.5489	44.02209	46.25024	45.41813	44.13535	52.1478	43.11819
414.6204	41.86188	45.54302	44.16825	43.30054	51.9189	41.57046
412.6919	40.77057	44.0994	41.94013	42.71239	50.60677	38.9948
410.7635	43.2561	44.03767	40.09679	40.91652	48.48848	37.16878
408.835	45.52435	42.49078	43.09317	40.03448	47.14611	37.10723
406.9065	41.53783	38.37252	41.65854	39.07136	44.70111	35.05545
404.9781	36.99456	36.50812	38.19944	35.96164	42.17339	30.84302
403.0496	34.73204	35.81807	36.34136	32.92056	40.26529	29.70587
401.1211	31.35265	33.42292	35.23619	30.75218	37.04565	26.60415

Appendix 33: Basic calculations absorbance values (540 nm-720 nm) for MTT assay: Dichloromethane-Ag NPs and Dichloromethane plant extract.

Wells	Concentration in µg/ ml	DCM AG NPS		Blank	DCM PLANT EXTRACT		Blank
A	0	0.3637	0.3983	0.09370	0.4148	0.4260	0.0091
B	0.14	0.2945	0.3245	0.08820	0.4054	0.4076	0.0036
C	0.28	0.3377	0.3676	0.1104	0.3677	0.3795	0.0059
D	0.42	0.2766	0.3786	0.1128	0.3376	0.3335	0.0055
E	3.7	0.3568	0.3676	0.1097	0.3026	0.3155	0.0081
F	11.11	0.3111	0.3727	0.1202	0.2573	0.2771	0.0074
G	33.33	0.2872	0.3189	0.1598	0.2493	0.2752	0.0037
H	100	0.02900	0.07450	0.1434	0.2426	0.1664	0.0020

Appendix 34: Basic calculations absorbance values (540 nm-720 nm) for MTT assay: aqueous-Ag NPs and aqueous plant extract.

Wells	Concentration in µg/ml	AQUEOUS Ag NPs			Blank	AQUEOUS PLANT EXTRACT		BlanK
A	0	1.3394	1.3490		0.093	0.4558	0.3922	0.07690
B	0.14	1.3152	1.3270		0.060	0.3842	0.3330	0.09310
C	0.28	1.3060	1.3220		0.093	0.3853	0.3561	0.09610
D	0.42	1.2907	1.2460		0.053	0.3879	0.3331	0.1062
E	3.7	1.2070	1.2160		0.070	0.4066	0.3391	0.1008
F	11.11	1.1986	1.1950		0.072	0.3546	0.2803	0.1075
G	33.33	0.1893	1.1799		0.037	0.3735	0.3212	0.07900
H	100	0.1549	1.1290		0.062	0.3083	0.3901	0.07920

Appendix 35: Basic calculations absorbance values (540 nm-720 nm) for MTT assay: dichloromethane-methanol-Ag NPs and dichloromethane-methanol plant extract.

Wells	Concentration in µg/ml	DCM MEOH PLANT EXTRACT		Blank	DCM MEOH Ag NPS		Blank
A	0	0.3636	0.3951	0.07720	1.408	1.419	0.061
B	0.14	0.3653	0.3577	0.1027	1.391	1.399	0.087
C	0.28	0.3745	0.3191	0.1066	1.378	1.392	0.060
D	0.42	0.3407	0.3216	0.1201	1.306	1.341	0.052
E	3.7	0.3577	0.3362	0.1233	1.299	1.308	0.048
F	11.11	0.3078	0.3234	0.1087	1.161	1.113	0.057
G	33.33	0.3274	0.3374	0.1038	1.129	1.092	0.046
H	100	0.3274	0.2988	0.1112	0.117	0.023	0.033

Appendix 36: Basic calculations absorbance values (540 nm-720 nm) for MTT assay: Methanol-Ag NPs and methanol plant extract

Wells	Concentration in µg/ml	MEOH Ag NPS		Blank	MEOH PLANT EXTRACT		Blank
A	0	0.4151	0.3318	0.07750	0.3087	0.2553	0.03150
B	0.14	0.3752	0.3375	0.07720	0.2959	0.2394	0.01980
C	0.28	0.4129	0.3221	0.1010	0.3132	0.1809	0.02000
D	0.42	0.4350	0.2731	0.1030	0.2647	0.3185	0.02260
E	3.7	0.3905	0.2995	0.09520	0.2886	0.3328	0.02440
F	11.11	0.3658	0.3762	0.09440	0.2835	0.2983	0.02020
G	33.33	0.3054	0.2685	0.01060	0.1933	0.2615	0.02280
H	100	0.1713	0.1271	0.08050	0.1279	0.1743	0.02040

Appendix 37: Basic calculations absorbance values (540 nm-720 nm) for MTT assay: dichloromethane- ethyl acetate Ag NPs and Dichloromethane- ethyl acetate plant extract

Wells	Concentration in µg/ml	DCM ETHLY ACETATE AG NPS		Blank	DCMETHLY PLANT EXTRACT Blank		Blank
A	0	0.4560	0.4718	0.0080	0.1868	0.2373	0.04150
B	0.14	0.4073	0.4056	0.0036	0.3305	0.3380	0.07860
C	0.28	0.3493	0.3315	0.0068	0.3695	0.3435	0.08590
D	0.42	0.3345	0.3277	0.0061	0.2983	0.2968	0.09370
E	3.7	0.3280	0.3203	0.0161	0.3662	0.3058	0.1006
F	11.11	0.3135	0.3158	0.0135	0.3566	0.2580	0.08090
G	33.33	0.2875	0.2680	0.0097	0.3271	0.3205	0.08600
H	100	0.1752	0.2542	0.0081	0.1739	0.2033	0.08870

Appendix 38: Basic calculations absorbance values (540 nm-720 nm) for MTT assay: Ethyl acetate-Ag NPs and Ethyl acetate plant extract

Wells	Concentration in µg/ml	ETHYL ACETTAE AGNPS		Blank	ETHYL ACETATE EXTRACT PLANT EXTRACT		Blank
A	0	1.392	1.411	0.069	0.3239	0.3955	0.1856
B	0.14	1.322	1.387	0.090	0.3156	0.3397	0.1022
C	0.28	1.311	1.308	0.052	0.3651	0.3922	0.1089
D	0.42	1.298	1.370	0.034	0.3787	0.3976	0.1198
E	3.7	1.253	1.360	0.038	0.3395	0.3739	0.1451
F	11.11	1.192	1.113	0.051	0.3904	0.3844	0.1216
G	33.33	1.159	1.106	0.053	0.3716	0.4233	0.1132
H	100	1.139	0.077	0.050	0.2917	0.2127	0.08900

Appendix 39: Basic calculations absorbance values (540 nm-720 nm) for MTT assay: silver nitrate and pure compounds as a mixture.

Wells	Concentration in µg/ml	SILVER NITRATE		Blank	PURE COMPOUNDS(MIXTURE)		Blank
A	0	0.3432	0.3692	0.0090	0.4048	0.4039	0.0081
B	0.14	0.3305	0.3245	0.0073	0.3807	0.4000	0.0041
C	0.28	0.2924	0.2879	0.0058	0.3644	0.3624	0.0095
D	0.42	0.2877	0.2766	0.0040	0.3506	0.3335	0.0087
E	3.7	0.2689	0.2938	0.0037	0.3226	0.3166	0.0057
F	11.11	0.2099	0.2056	0.0035	0.3112	0.3085	0.0042
G	33.33	0.1618	0.1485	0.0025	0.2327	0.2718	0.0098
H	100	0.0570	0.0081	0.0010	0.1857	0.1970	0.0095

Appendix 40: Basic calculations absorbance values (540 nm-720 nm) for MTT assay for doxorubicin

Wells	Concentration in µg/ml	DOXORUBICIN		Blank
A	0	0.051	0.7046	1.563
B	0.14	0.038	1.027	1.554
C	0.28	0.034	1.164	1.466
D	0.42	0.052	1.394	1.72
E	3.7	0.09	0.6081	1.285
F	11.11	0.069	0.8948	1.089
G	33.33	1.066	0.8526	1.309
H	100	1.3	1.039	1.238

Appendix 41: Puplication 1

Full Length Research Paper

Anti-bacterial activity of secondary metabolites from *Chrysanthemum cinerariaefolium*

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This study evaluated antibacterial activity of *Chrysanthemum cinerariaefolium* (pyrethrum) flower dichloromethane crude extract, fractions and isolated compounds; pyrethrin II, jasmolin I and cinerolone against *methicillin-resistant Staphylococcus aureus (MRSA), Pseudomonas aeruginosa, Staphylococcus aureus* and *Shigella sonnei.* The isolated compounds were obtained by carrying out column chromatography on dichloromethane extract and purifying the fractions using preparative High Perfomance Liquid Chromatography (HPLC). The structures of the isolated compounds were elucidated using 1D and 2D NMR. The bioactivity of crude extract, fractions and isolated compounds were determined using disc diffusion assay at a concentration of 100 mg/mL. The MIC and MBC were determined using microdilution method. The bioassay results showed that individually isolated compounds were not active on all the micro-organisms except Jasmolin I which showed slight activity on *P. aeruginosa* with 7.7±0.6 mm. There was significant difference in the activity of the isolated compounds as a mixture and the activity of individual compounds on MRSA, *S. aureus*, *P. aeruginosa*, with P= 0.01, P= 0.0002, P= 0.0007 respectively (α =0.05, Tukey's test). Isolated compounds and isolated compounds as a mixture in a ratio of (1:1:1) were not active on *S. sonnie.* Those fractions and isolated compounds which caused inhibition zones of above 10 mm were subjected to MIC and MBC. The lowest MIC and MBC observed was for fraction 3 against MRSA which were 6.5 and 12.5 mg/mL respectively. The compound mixture had MIC and MBC of 25 and 50 mg/ml respectively against *P. aeruginosa.*

Key words: MIC (minimum inhibitory concentration), MBC (minimum bacteriostatic concentration), bioassay, *Chrysanthemum cinerariaefolium*, Jasmolin, Pyrethrin II, Cinerolone.

Appendix 42: Publication 2

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Synthesis of Silver Nanoparticles Using Dichloromethane Extract of Chrysanthemum cinerariaefolium and Its Bioactivity

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ABSTRACT

Common methods of synthesizing metallic nanoparticles are chemical and physical. However, they are expensive and use toxic chemicals. Green synthesis is less costly and safer, hence a potential alternative. Silver nanoparticles (Ag NPs) were synthesized using dichloromethane extract of Chrysanthemum cinerariaefolium, and colour change from pale green to dark brown was observed. Scanning electron microscopy (SEM) images were faceted, and others formed clusters. Transmission electron microscopy (TEM) images were spherical with an average size of 22.8± 17.5 nm. EDX analysis showed the nanoparticles had percentage abundance of 67.26%. Fourier-transform infrared spectroscopy (FTIR) analysis showed absorption bands at 3489.59 cm-1, 3217.80 cm-1, 2384.74 cm-1, 1633.05 cm-1, 1405.08 cm-1, 1109.32 cm-1, and 505.93 cm-1. The UV-Vis analysis showed surface plasmon resonance (SPR) peak at 434 nm. The nanoparticles were more active on P. aeruginosa with an MIC of 15 µg/ml while the cytotoxicity assay showed Ag NPs had an MIC of 33.33 µg/ml, and hence, were noncytotoxic against Vero cells.

KEYWORDS

Bioassay, Characterization, Cytotoxicity, Nanoparticles, Synthesis

Appendix 43: Publication 3

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Silver Nanoparticles Using Dichloromethane-Methanol Flower Extract of *Chrysanthemum cinerariaefolium* and Its Antibacterial Activity

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Abstract: Nanotechnology is an emerging field that has opened new horizons in nanomedicine. The use of silver nanoparticles is attracting much interest because of their antibacterial activity. This study involved synthesis of silver nanoparticles using *Chrysanthemum cinerariaefolium* flowers dichloromethane-methanol crude extract. The synthesized silver nanoparticles (Ag NPs) were characterized using UV-Vis spectroscopy, SEM, EDX, TEM and FTIR. The antibacterial potential of the nanoparticles was ascertained against *methicillin-resistant Staphylococcus aureus (MRSA), Pseudomonas aeruginosa, Staphylococcus aureus* and *Shigella sonnei*. This was followed by phytochemical analyses of the crude extracts. The Ag NPs were generally spherical as observed in the SEM and TEM micrographs with an average size of 26.98 nm. The UV- absorption spectrum revealed prominent peak at 430 nm while EDX analysis showed the percentage abundance of silver nanoparticle at (81.33%). The FTIR spectroscopy confirmed absorption bands of various functional groups on the surface of Ag NPs. The absorption bands were at 3472.88 cm^{-1}, 3190.67 cm^{-1}, 1646.61 cm^{-1}, 1405.08 cm^{-1}, 1109.32 cm^{-1} and 518.64 cm^{-1}. Antibacterial potential of the synthesized Ag NPs showed that they were more active on *S. aureus* with an MIC of 31.25 µg/ml. The phytochemicals observed in the crude extracts that could have been responsible for reducing silver ions into silver nanoparticles were flavonoids, phenols, tannins and glycosides.

Keywords: Nanoparticles, Nanotechnology Antibacterial, Phytochemicals