Synthesis, Characterization and Evaluation of Novel Treatment against Resistant Pathogenic Bacteria

BY

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To

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DECLARATION

I, Arinao Murei – student number: 11635633 do hereby declare that this dissertation is the results of my own research, that it does not incorporate without acknowledgement any material. It has not been submitted for a degree or diploma at any university and does not contain any materials previously published, written or produced by another person except where due reference is made in the text.

Signed (Student): ..... Date: .....

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DEDICATIONS

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
µl	Microliter
AgNP	Silver nanoparticles
ATCC	American Type Culture Collection
ATPase	Adenosine triphosphatase
AuNP	Gold nanoparticle
CFU	Colony-forming unit
DPPH	1.1-diphenyl-2-picrylhydrazyl
EDX	Energy dispersive X-ray analysis
FTIR	Fourier-transform infrared spectrometry
g	Gram
HAuCl4	Chloroauric acid
HRTEM	High resolution transmission electron microscopy
IC_{50}	50% Inhibitory Concentration
INT	p-iodonitrotetrazolium chloride
MBC	Minimum bactericidal concentration
mgGA/g	Milligram Gallic acid equivalent per gram
mgQE/g	Milligram equivalent of quercetin per gram
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MIC	Minimum inhibition concentration
ml	Milliliter
mm	Millimeter
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-Susceptible *Staphylococcus aureus*
NaBH4	Sodium borohydride

nm	Nanometer
OD	Optical density
PEG	Polyethylene glycol
Rpm	Revolutions per minute
SA	South Africa
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
UV	Ultraviolet
Vis	Visible
WHO	World Health Organisation

PUBLICATIONS EMANATING FROM THE RESEARCH

The following manuscripts have been developed from the study, with one being published and the others are in final stage of preparation.

Samie, A., Murei, A. and Ramalivhana, J.N., 2017. Evaluation of Antimicrobial Activities of Extract from *Pyrenacantha grandiflora* Baill.(Icacinaceae). Pakistan Journal of Biological Sciences, 20, pp.498-506.

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Murei, A., Pillay, K., Govender, P., and Samie, A., Synthesis, Characterization and *In Vitro* Antibacterial Evaluation of *Pyrenacantha grandiflora* Conjugated Silver Nanoparticles. To be submitted to Journal of Photochemistry & Photobiology.

Murei, A., Samie, A., and Pillay, K., Synthesis, Characterization and Evaluation of Antibacterial Activity of *Pyrenacantha grandiflora* Conjugated with Gold Nanoparticles. To be submitted to Digest Journal of Nanomaterial and Biostructure.

Murei, A., and Samie, A., Functionalization of Penicillin, Vancomycin and Ampicillin with *Pyrenacantha grandiflora* Baill and Silver Nanoparticles. To be submitted to Advanced Pharmaceutical Bulletin.

ABSTRACT

BACKGROUND: Antibiotic resistance amongst microbial pathogens has become a challenge over past decades, bringing about genuine and frequently deadly contaminations that can't be dealt with by ordinary means. This has led to a search on developing solutions to this problem by searching for new source of antimicrobial agents or chemically altering the existing ones. Traditional medicinal plants and nanoparticles are highly targeted as promising agents to address the challenge. *Pyrenacantha grandiflora Baill* from Icacenaceae family possess pharmaceutical activities and is used by Vhavenda people to cure gastrointestinal related infections, diarrhea and tooth pain.

OBJECTIVES: The present study aimed to synthesize, characterize and evaluate the efficacy of *Pyranacantha grandiflora* extracts alone and when conjugated with selected nanoparticles against pathogenic microorganisms. Furthermore, this study investigated the efficacy of selected antibiotics when conjugated with nanoparticles against selected pathogenic microbes.

METHODS: *Pyrenacantha grandiflora* Baill (tubers) were collected from Masisi area. Bioactive compounds were extracted using different solvents such as methanol, acetone, hot water, dichloromethane and chloroform. Preliminary phytochemical screening was done to identify different phytochemicals in the extracts and their functional groups were identified by Fourier Transform Infrared spectroscopy (FTIR). Extracts were further assessed for their total phenolic and flavonoids content. Thin layer chromatography was used to separate the compounds from the plant extracts and active compounds/group of compounds were identified by bioautography. The antioxidant ability of the extracts to scavenge free radical DPPH was also determined. Silver and gold nanoparticles were synthesized using chemical and biological methods, characterized by Ultraviolet-Visible Spectrophotometry (UV-VIS), Transmission Electron Microscopy (TEM) and Energy dispersive X-ray analysis (EDX). Plant extracts, nanoparticles and antibiotics were

conjugated differently, and conjugates were analyzed by FTIR and their antimicrobial activities were evaluated against different bacteria and fungi. The conjugates were tested for antimicrobial activity against extended *beta*-lactamase producing *Escherichia coli* (ATCC 35218), *Escherichia coli* (ATCC 25922), methicillin-resistant *Staphylococcus aureus* (ATCC 25923), methicillin-susceptible *Staphylococcus aureus* (ATCC 33591) and *beta*-lactamase producing *Klebsiella pneumonia* (ATCC 700603) using agar diffusion assay and the minimum inhibitory concentrations (MIC) were determined using the microdilution method. The minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were determined by sub-culturing from the MIC plates on Mueller-Hinton Agar.

RESULTS: *Pyrenacantha grandiflora* was found to contain phenolics, saponins, alkaloids, tannin, steroids, terpenoids and flavonoids. FTIR spectroscopic studies revealed different characteristic peak values with various functional compounds similar in most extracts but differed with transmittance values. The total phenolic contents in the examined extracts ranged from 14.167 to 19.02 mg GA/g. The total flavonoid content in the examined extracts ranged from 26.603 to 34.621 mg QE/g. Thin-layer chromatography revealed various R_f values and when analyzed with bioautography, well-defined inhibition zones within the R_f value of 0.236 was identified against *E. coli* and *K. pneumonia*. The MICs of the extracts were determined, and all the extracts showed some antimicrobial activity against all tested strains ranging from 0.06-7.5 mg ml/g. Some extracts appeared to be fungicidal and hot water extracts were more active against *Cryptococcus neoformans* with the MFC value of 0.06 mg/ml. The methanol extract was also active against most tested strains including *Candida tropicalis* with the minimum fungicidal concentration value of 3.75 mg/ml. *Pyrenacantha grandiflora* tuber extracts conjugated with silver or gold nanoparticles exhibited a good antibacterial activity against all bacterial strain used and very few were able to exhibit bactericidal activity. Penicillin showed improvement of antibacterial activity

when conjugated with compounds from the acetone extracts and vancomycin was found to be more effective when conjugated with silver nanoparticles and water extracts.

CONCLUSION: The present study validated the efficacy of conjugated *P. grandiflora* tuber extracts which is used in traditional medicine. The results revealed that water extracts which are generally used by the traditional healers are active against most microorganisms tested as well as methanol and acetone extracts and the synergistic effect was observed when they were conjugated to gold and silver nanoparticles. The results of the present investigation clearly indicate that antimicrobial activity of *Pyrenacantha grandiflora* Baill tuber when conjugated with selected nanoparticles and antibiotics vary with test strain and the type of solvent used during extraction, thus giving hope for future development of drug leads.

Keywords: Antibiotic resistance, Phytochemical, Nanoparticles, Antimicrobial activity, Minimum inhibition concentration

CHAPTER ONE: INTRODUCTION

1.1 BACKGROUND

Antimicrobial resistance has become a major challenge that the world is facing, and it is threatening our capacity to treat regular ailments, bringing about disease, disability and death (WHO, 2016). In 1928 when the first antibiotic (penicillin) was discovered, bacteria started developing a mechanism of resistance against different synthetic antibiotics (Jason *et al*., 2016). This is yet a more prominant concern since new resistance mechanisms are developing and spreading worldwide. Strains of *Staphylococcus aureus, Escherichia coli a*nd *Klebsiella pneumoniae* are the causative agents of critical diseases in and out of hospitals and have evolved drug resistance to many of the established antimicrobial compounds on the market at present (Brown *et al.,* 2012; O'Bryan *et al*., 2018). Research has been focused on developing several strategies to this issue by making novel antibacterial agents or artificially adjusting the action of current cost effective antibiotics (Tillotson and Theriault, 2013). Traditional medicinal plants and nanoparticles are targeted as promising agents that could be used to address the antibiotic resistance challenge due to their efficacy as antimicrobial agents.

Initially plants have been utilized by the general population to meet their nutritional necessities and some vegetation (depending on their characteristics) turned into extremely helpful substances to cure for a wide range of illnesses across different communities (Duraipandiyan *et al*., 2006; Malik *et al*., 2005; Mustafa *et al.*, 2017). Many various plant species are still in use in many parts of the world as they contain bioactive compounds responsible for healing purposes (Nostro *et al*., 2000). Secondary metabolites of plants such as terpenoid, alkaloids and phenolic compounds are mostly known to possess antimicrobial activities (Savoia, 2012; Mahato and Sen, 1997; Kappers *et al*., 2005). Medicinal plants are guaranteed to be a standout amongst the most encouraging sources because of their natural origin and biocompatibility as compared to the

synthetic compounds (Rajeh *et al*., 2010; Upadhyay *et al*., 2014). Several studies have been done in South Africa to highlight antibacterial activities of some medicinal plants (Samie *et al*., 2007; Gundidza *et al*., 2009). *Pyrenacantha grandiflora* Baill is traditionally used in the Venda region to treat and manage diarrhea. Moreover, their antimicrobial activity has been investigated and shows to be effective against numerous disease-causing microorganisms (Ramalivhana, 2010; Samie *et al*., 2007). Therapeutic plants have a promising future and there are approximately half a million plants far and wide. However, forthe majority of these plants, their medicinal activity has not been explored yet, although their restorative activities could be unequivocal in the treatment of present or future diseases (Rasool Hassan, 2012). Scientists and clinicians need to strive to illuminate the important active components which can be separated from therapeutic plants. Also, research need to clarify the part of plant which is active in the treatment of present illnesses, and how they can be utilized to deliver more successful medications.

Over the past few years, a wide range of nanoparticles engineered from different nanomaterials have been created via innovative synthetic techniques, yielding a plethora of unique properties and potential applications (Parveen *et al*., 2012). Furthermore, nanoparticles have numerous favorable properties, which include the ability to self-assemble, high stability in biological systems, specificity with regards to tissue targeting, the ability to encapsulate drugs and providing image contrast for visualization purposes (Gindy and Prud'homme, 2009). Recent studies involving metal nanoparticles show that they possess antimicrobial activity and thus have a potential for enhancement of treatment of infections, if properly adapted into existing procedures (Giljohann, 2010; Abbasi *et al*., 2014; Pereira *et al*., 2015, Rai *et al*., 2016). Thus, for resistance to develop against the nanoparticles, a great number of synchronized mutations amongst these species would have to occur (Huh and Kwon, 2011).

Amongst all the nanoparticles, gold and silver nanoparticles have increased consideration for the outline and improvement of inventive biomedical tools (Drbohlavova *et al*., 2013; Jiang *et al*.,

2013; Kumar *et al*., 2013; Zhao and Jiang, 2013; Shah *et al*., 2014). Regardless of the way that gold and silver nanoparticles are strong candidates for antibacterial specialists, gold nanoparticles have been more broadly used as nanoparticles for cancer treatment (Ahmad *et al*., 2013; Drbohlavova *et al*., 2013; Almeida *et al*., 2014), while less broad endeavors have been made in the field of antibacterial agents (Zhao and Jiang, 2013). Nanoparticles, particularly gold and silver nanoparticles conjugated with antibiotics may subsequently be utilized to address the issue related to ordinary antibiotic treatment (Bhattacharya, 2012). Therefore, this MSc research study has evaluated antibacterial activity of *P. grandiflora* tuber extracts conjugated gold and silver nanoparticles as well as antibiotics (ampicillin, penicillin and vancomycin) known to have limited efficacy on their own. In addition, plant extracts conjugated with the nanoparticles were screened against *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumonia* that were chosen based on their widespread and common occurrence as disease-causing microbial agents in both the community and nosocomial settings (Zetola *et al*., 2005).

1.2 PROBLEM STATEMENT

Antibiotic-resistant strains of bacteria have emerged following the increased use of antibiotics and their integration (Wood *et al*., 1996; Walsh, 2000). In addition to the constant development of resistance, the genes coding for resistance to antibiotics are not easily lost once evolved in a bacterial population. Upon integration, these genes become a stable component of the bacterial genome. As further resistance mechanisms are developed, it becomes harder to provide treatment that can effectively combat this multi-drug resistance phenomenon (Livermore, 2003). Most of the therapeutic agents that are used to date are synthesized from plants. Furthermore, some of these are endangered species (Braun and Pitt, 2018) and very soon they will run-out resulting in search of the new source of medicine. These new research foci need to be directed

towards creating a long-term solution to these resistance issues, one that can, and will, be implemented as a framework for future medical work in these areas (Taylor *et al*., 2002).

1.3 RATIONALE OF THE STUDY

Antibiotics are currently being rendered obsolete by the rising incidence of drug resistance amongst pathogenic microorganisms and infections are becoming untreatable (Ventola, 2015). In 2007 Clatworthy *et al*. revealed that most antibiotics target only a few cellular processes in the bacteria, and this limited the spectrum of target which contributes to the easy and rapid development of antibiotic resistance. Drug resistance and re-emerging diseases are challenges which modern medical research and drug development seek to address (Ventola, 2015). Some bacteria are naturally resistant to antibiotics, while others acquire resistance by a different mechanism such as mutation in the genes, acquisition of new genetic elements encoding for defensive enzymes and proteins (Clatworthy *et al*., 2007). For example, mutation in DNA gyrase enzyme results in resistance towards quinolones in *Escherichia coli* and *Staphylococcus aureus,* and *Klebsiella pneumonia*, and *S. aureus* can develop resistance against vancomycin (Chang *et al*., 2003). Mutations affecting RNA polymerase *β* subunit have resulted in resistance of *E. coli* towards rifampicin (Garbisu *et al*., 2018). Subsequently, enhancements to existing treatments and the development of novel medicines are desperately required to manage this heightening risk to human health. Nanoparticles and traditional medicinal plants in this study are promising strategies to combat antibiotic resistance.

Medicinal plants have been used for many years in the treatment of human diseases and many studies show their effectiveness as antimicrobial agents. From a previous study, *P. grandiflora* has been evaluated for antimicrobial activity and was shown to be effective against *Aeromonas* spp, but no study has been done to identify its active compounds that are responsible for killing

or inhibiting the growth of the microorganisms (Obi, 2010). Thus, this research study has identified phytochemicals of *P. grandiflora*. Nanoparticles are also known to be effective against microorganisms and gold nanoparticles have gained enormous consideration as biomedical tools due to their similarity with biological materials (Parveen *et al*., 2012). No resistance has been revealed with gold nanoparticles which makes them good candidates to resolve this issue of microbial resistance. Thus, for resistance to develop against the nanoparticles, a great number of synchronized mutations amongst these species would have to occur (Huh and Kwon, 2011).

To develop a novel treatment, two or more effective treatment options can be combined. In most studies, an increase in antimicrobial activity was reported after conjugation of the antibiotic to the nanoparticle and gold nanoparticles was reported to improve antimicrobial activity of ciprofloxacin. Formulations from the different medicinal plants are also very interesting in curing multiple infections and symptoms. For example, Iberigast, a medicine that harnesses the power of nine natural plant extracts to relieve multiple digestive symptoms such as abdominal pain and cramps, fullness, heaviness, bloating, flatulence and nausea (Raedsch *et al*., 2018). Several studies have been done to highlight the efficacy of gold and silver nanoparticle co-synthesized from plant extracts. However, no study has ever reported on the non-covalent interaction of *P. grandiflora* extracts with gold nanoparticle nor silver nanoparticles. Hence, this is the first study to report conjugation of *P. grandiflora* with nanoparticles and antibiotics.

Antibiotics such as penicillin, ampicillin and vancomycin are rendered absolete since they have lost their efficacy in curing common illnesses. Scientists are however still trying to enhance the activity of less active antibiotics by co-synthesis of nanoparticles with those antibiotics. These antibiotics have been conjugated to nanoparticles and led to increased antimicrobial activity against multidrug resistant microorganisms including methicillin-susceptible and methicillin-resistant forms of *Staphylococcus aureus* (Turos *et al*., 2007). However, those nanoparticles were chemically synthesized. This study uses a green synthetic strategy for nanoparticles production,

using the *Magnetospirillium* bacterium and conjugated them to plant extracts and antibiotics. The co-existence of two different molecules with entirely different modes of action in the form of hybrid molecules has been reported to produce a synergistic effect and thus this study has evaluated the effectiveness of *P. grandiflora* extract conjugated with gold and silver nanoparticles as well as antibiotics in order to offer advantages such as dosage compliance, minimizing toxicity and overcoming drug resistance when compared to the parent counterparts.

1.4 OBJECTIVES OF THE STUDY

1.4.1 Broad objective of the study

- To synthesize, characterize and evaluate the antimicrobial activity of *Pyrenacantha grandiflora* and its conjugant to selected nanoparticles and antibiotics.

1.4.2 Specific Objectives of the Study

- To conduct the preliminary phytochemical screening of *P. grandiflora*.
- Identification of *P. grandiflora* functional groups using Fourier-transform infrared spectroscopy.
- Investigatation of the phytochemical content and antioxidant activity of extract from *P. grandiflora* tubers.
- Chemical and biological synthesis of gold and silver nanoparticles.
- Characterization of nanoparticles using UV-VIS spectrophotometry, TEM and EDX.
- Non-covalent conjugation of gold and silver nanoparticles to plant extracts.
- Conjugation of antibiotics and plant extracts to nanoparticles.
- Analysis of conjugates using FTIR.

- Determination of the antimicrobial activity of *P. grandiflora* extracts by agar diffusion assay and broth microdilution method.
- Determination of the fractional inhibition concentration index (FICI) of *P. grandiflora* conjugated with synthesized nanoparticles and antibiotics.

1.5 HYPOTHESES

In the present study, we hypothesize that *Pyrenacantha grandiflora* tubers extract contain important phytochemicals that have good antioxidant activity. The effectiveness of plant extracts is enhanced when conjugated with either nanoparticles or antibiotics. All the conjugate work effectively at low concentrations.

1.6 *REFERENCES*

Abbasi, E., Milani, M., Fekri Aval, S., Kouhi, M., Akbarzadeh, A., Tayefi Nasrabadi, H., Nikasa, P., Joo, S.W., Hanifehpour, Y., Nejati-Koshki, K. and Samiei, M., (2016). Silver nanoparticles: synthesis methods, bio-applications and properties. *Critical reviews in microbiology*, 42(2), pp.173-180.

Abtahi, S.M.H., (2013). Synthesis and characterization of metallic nanoparticles with photoactivated surface chemistries (Doctoral dissertation, Virginia Tech).

Ahmad, M.Z., Akhter, S., Rahman, Z., Akhter, S., Anwar, M., Mallik, N. and Ahmad, F.J., (2013). Nanometric gold in cancer nanotechnology: current status and future prospect. *Journal of Pharmacy and Pharmacology*, 65(5), pp.634-651.

Almeida, J.P.M., Figueroa, E.R., and Drezek, R.A., (2014). Gold nanoparticle mediated cancer immunotherapy. *Nanomedicine* 10, 503–514.doi: 10.1016/j.nano.2013.09.011.

Bhattacharya, I., (2012). Evaluation of antioxidant capacityand phenol content of five different vegetables from kolkata. Barrackpore Rastraguru Surendranath College (Masters dissertation).

Boucher, H.W., Bakken, J.S. and Murray, B.E., (2016). The United Nations and the Urgent Need for Coordinated Global Action in the Fight against Antimicrobial Resistance Coordinated Global Action to Fight against Antimicrobial Resistance. *Annals of Internal Medicine*, 165(11), pp.812-813.

Braun, B. and Pitt, R., (2018). Homeopathy as a Means of Conserving Endangered Medicinal Plant Species: A Homeopathic Proving of an Important Herbal Medicine in Southern Africa. *Homeopathy*, *107*(S 01), p.P003.

Brown, A.N., Smith, K., Samuels, T.A., Lu, J., Obare, S.O. and Scott, M.E. (2012). Nanoparticles Functionalized with Ampicillin Destroy Multiple-Antibiotic-Resistant Isolates of *Pseudomonas aeruginosa* and Enterobacter aerogenes and Methicillin-Resistant *Staphylococcus aureus*. *Applied and environmental microbiology*, 78, 2768-2774.

Chang, S., Sievert, D.M., Hageman, J.C., Boulton, M.L., Tenover, F.C., Downes, F.P., Shah, S., Rudrik, J.T., Pupp, G.R., Brown, W.J. and Cardo, D., (2003). Infection with vancomycin-resistant *Staphylococcus aureus* containing the vanA resistance gene. *New England Journal of Medicine*, 348(14), pp.1342-1347.

Clatworthy, A.E., Pierson, E. and Hung, D.T., (2007). Targeting virulence: a new paradigm for antimicrobial therapy. *Nature chemical biology*, 3(9), p.541.

Drbohlavova, J., Chomoucka, J., Adam, V., Ryvolova, M., Eckschlager, T., Hubalek, J. and Kizek, R., (2013). Nanocarriers for anticancer drugs-new trends in nanomedicine. *Current drug metabolism*, 14(5), pp.547-564.

Duraipandiyan, V., Ayyanar, M. and Ignacimuthu, S., 2006. Antimicrobial activity of some ethnomedicinal plants used by Paliyar tribe from Tamil Nadu, India. *BMC complementary and alternative medicine*, 6(1), p.35.

Garbisu, C., Garaiyurrebaso, O., Lanzén, A., Álvarez-Rodríguez, I., Arana, L., Blanco, F., Smalla, K., Grohmann, E. and Alkorta, I., (2018). Mobile genetic elements and antibiotic resistance in mine soil amended with organic wastes. *Science of The Total Environment*, 621, pp.725-733.

Giljohann, D.A., Seferos, D.S., Daniel, W.L., Massich, M.D., Patel, P.C. and Mirkin, C.A., (2010). Gold nanoparticles for biology and medicine. *Angewandte Chemie International Edition*, 49(19), pp.3280-3294.

Gindy, M.E. and Prud'homme, R.K., (2009). Multifunctional nanoparticles for imaging, delivery and targeting in cancer therapy. *Expert opinion on drug delivery*, 6(8), pp.865-878.

Gundidza, M., Gweru, N., Mmbengwa, V., Ramalivhana, N.J., Magwa, Z. and Samie, A., (2009). Phytoconstituents and biological activities of essential Oil from *Rhus lancea* L. F. *African Journal of Biotechnology* 7 (16): 2787-2789.

Huh, A.J. and Kwon, Y.J. (2011). Nanoantibiotics‖: A new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era. *Journal of Controlled Release*, 156, 128-145.

Jason, N., Payne, H.K., Waghwani, M.G., Connor, W.H., Tockstein, S., Moolani, H., Chavda, F., Badwaik, V., Matthew, B.L. and Rajalingam D., (2016). Novel Synthesis of Kanamycin Conjugated Gold Nanoparticles with Potent Antibacterial Activity. *Forntier in Microbiology*. doi: 10.3389/fmicb.2016.00607.

Jiang, Z.H. and Koganty, R., (2003). Synthetic vaccines: the role of adjuvants in immune targeting. *Current medicinal chemistry*, 10, 1423-1439.

Kappers, I.F., Aharoni, A., Van Herpen, T.W., Luckerhoff, L.L., Dicke, M. and Bouwmeester, H.J., (2005). Genetic engineering of terpenoid metabolism attracts bodyguards to Arabidopsis. *Science*, 309(5743), pp.2070-2072.

Kumar, S.A., Chang, Y.T., Wang, S.F. and Lu, H.C., (2010). Synthetic antibacterial agent assisted synthesis of gold nanoparticles: Characterization and application studies. *Journal of Physics and Chemistry of Solids*, 71, 1484-1490.

Livermore, D.M., (2003). Bacterial resistance: origins, epidemiology, and impact. Clinical *Infectious Diseases*, 36, S11-S23.

Mahato, S.B., Sen, S., (1997). Advances in triterpenoid research, 1990-1994. *Phytochemistry* 44: 1185-1236.

Malik, F., Hussain, D., Dil A.S., Hannan, A., Gilani. A.H., (2005). WHO Global Atlas of Traditional, Complementary and Alternative Medicine, Islamic republic of Pakistan. In: Ong, C.K., G. Bodeker, C. Grundy, G. Burford and K. Shein. (Eds.). (Map Volume). World Health Organization. Geneva. pp. 165–169 (Chapter22).

Mustafa, G., Arif, R., Atta, A., Sharif, S. and Jamil, A., (2017). Bioactive compounds from medicinal plants and their importance in drug discovery in Pakistan*. BMC complementary and alternative medicine*. 18. pp. 851-865.

Nostro, A., Germano, M.P., D'angelo, V., Marino, A. and Cannatelli, M.A., (2000). Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. *Letters in applied microbiology,* 30(5), pp.379-384.

Parveen, S., Misra, R. & Sahoo, S.K. (2012). Nanoparticles: a boon to drug delivery, therapeutics, diagnostics and imaging. *Nanomedicine: Nanotechnology, Biology and Medicine*, 8, 147-166.

Pereira, L. F., Mehboob, A., JM Stams. (2015) Metallic nanoparticles: microbial synthesis and unique properties for biotechnological applications, bioavail-ability and biotransformation. *Critical Review Biotechnology*. 35, 114–128.

Raedsch, R., Vinson, B., Ottillinger, B. and Holtmann, G., (2018). Early onset of efficacy in patients with functional and motility-related gastrointestinal disorders. *Wiener Medizinische Wochenschrift*, 168(3-4), pp.89-98.

Rai , M., Ingle, A.P., Birla, S., Yadav, A. and Santos, C.A.D., (2016). Strategic role of selected noble metal nanoparticles in medicine. *Critical reviews in microbiology*, 42(5), pp.696-719.

Rajeh, M.A.B., Zuraini, Z., Sasidharan, S., Latha, L.Y. and Amutha, S., (2010). Assessment of Euphorbia hirta L. leaf, flower, stem and root extracts for their antibacterial and antifungal activity and brine shrimp lethality. *Molecules*. 15:6008–18.

Ramalivhana, N. J. (2010). Molecular characterization of Aeromonas hydrophila and antimicrobial activities of selected medicinal plants against pathogenic isolates from water and stool samples in the era of HIV/AIDS in Limpopo Province, South Africa (Doctoral dissertation).

Rasool Hassan Bassam Abdul, (2012).Medicinal plants (Importants and uses). *Pharmaceutical Analytica Acta* 3: e139. doi:10.4172/2153-2435.1000e139.

Samie, A., Ramalivhana, J., Igumbor, E.O. and Obi, C.L., (2007). Prevalence, Haemolytic and haemagglutination activities and antibiotic susceptibility profiles of *Campylobacter spp*. isolated from diarrhoeal stools in Vhembe district, South Africa. Journal of Health Population and Nutrition 25(4): 406-413.

Savoia, D., (2012). Plant-derived antimicrobial compounds: alternatives to antibiotics. *Future microbiology*, 7(8), pp.979-990.

Shah, M., Badwaik, V., Kherde, Y., Waghwani, H.K., Modi, T., Aguilar, Z.P., (2014).Gold nanoparticles: various methods of synthesis and antibacterial applications. *Frontiers in Bioscience*.19:1320. doi: 10.2741/4284.

Tillotson, G.S., and Theriault, N., (2013). New and alternative approaches to tackling antibiotic resistance. *F1000 Prime Reports.* 5:51. doi: 10.12703/P5-51.

Turos, E., Reddy, G. S. K., Greenhalgh, K., Ramaraju, P., Abeylath, S. C., Jang, S., and Lim, D. V. (2007). Penicillin-bound polyacrylate nanoparticles: restoring the activity of *β*-lactam antibiotics against MRSA. *Bioorganic & medicinal chemistry letters*, 17(12), 3468-3472.

Upadhyay, A., Upadhyaya, I., Kollanoor-Johny, A. and Venkitanarayanan, K., (2014). Combating Pathogenic Microorganisms Using Plant-Derived Antimicrobials: A Minireview of the Mechanistic Basis. *BioMed Research International*. 2014:18.

Ventola, C. L. (2015). The antibiotic resistance crisis: part 1: causes and threats. *Pharmacy and Therapeutics*, 40(4), 277.

Walsh, C., (2000). Molecular mechanisms that confer antibacterial drug resistance. *Nature*, 406, 775-781.

Wang, H., Chen, Y., Li, X. Y., & Liu, Y. (2007). Synthesis of oligo (ethylenediamino)-*β*-cyclodextrin modified gold nanoparticle as a DNA concentrator. *Molecular pharmaceutics*, 4(2), 189-198.

Wood, A.J., Gold, H.S. and Moellering, J.R, (1996). Antimicrobial-drug resistance. *New England Journal of Medicine*, 335, 1445-1453.

World Health Organization. (2016). Antimicrobial resistance. *Journal of Global Antimicrobial Resistance surveillance.* Geneva: WHO Press.

Zetola, N., Francis, J. S., Nuermberger, E. L., & Bishai, W. R. (2005). Community-acquired meticillin-resistant *Staphylococcus aureus*: an emerging threat. *The Lancet infectious diseases*, 5(5), 275-286.

Zhao, Y., and Jiang, X., (2013). Multiple strategies to activate gold nanoparticles as antibiotics. *Nanoscale* 5, 8340–8350.doi:10.1039.

CHAPTER TWO: LITERATURE REVIEW

2.1 BACKGROUND ON MEDICINAL PLANTS

Medicinal plants are used in many different parts of the world as major therapeutic agents since they contain bioactive compounds responsible for healing (Nostro *et al*., 2000). Initially plants have been utilized by the general population to meet their nutritional necessities and characteristic vegetation turned into an extremely helpful hotspot for health change and to cure a wide range of illnesses in different comunities (Duraipandiyan *et al*., 2006; Malik *et al*., 2005; Mustafa *et al.*, 2017). Medicinal plants include different types of plants utilized as part of herbalism and are considered as a rich resource of ingredients which can be utilized as part of medication advancement or synthesis (Rasool Hassan, 2012). In addition, the greater part of pills and capsules in our market originate from plants. Medicinal plants can be utilized as crude materials for extraction of active compounds that are utilized in amalgamation of the various drugs. Secondary metabolites such as terpenoid, alkaloids and phenolic compounds are known to be active compounds of plants and are extracted as phytochemicals. Hence, they are mostly known to be responsible for antimicrobial action (Savoia, 2012; Mahato and Sen, 1997; Kappers *et al*. 2005).

There are approximately half a million plants worldwide, and for the vast majority of them, their medicinal activity has not yet been explored, and their restorative exercises could be unequivocal in the treatment of present or future illnesses (Rasool Hassan, 2012). A few reports, on the antibacterial activity of medicinal plants against pathogenic bacteria have been conducted (Samie *et al*., 2007; Eloff *et al*., 2005). Different solvents that have been employed in plant extraction include methanol, water, ethyl acetate, acetone and chloroform. Different plant tissues or whole plants are used in treatment of different ailments. Different parts of medicinal plants are found in

markets, and they have been identified as, roots (61%), entire plant (22%), barks (15%), foods grown from the ground (1%), and leaves (1%) (Tshisikhawe, 2002).

2.1.1 Phytochemicals of plants extract

Phytochemicals are non-nutritive substances from plants that have defensive or illness preventive properties (Alachaher *et al*., 2018). They are non-nutritive supplements, denoting that they are not needed by the people for supporting life. There are many different phytochemical compounds which include chlorophyll, alkaloids, terpenoid, and phenolic acids and others (secondary constituents) (Krishnaiah, 2007). Different plant tissue has been screened to identify phytochemicals and some examples are shown in **Table 2.1** (Wangchuk, 2014). It is known that every plant has these phytochemicals at different proportions to protect them against infection, but literature has revealed that they also protect humans against infections (Krishnaiah, 2007). For example, dried berries possess active compounds such as tannins, flavones and glycosides, and are mostly used as an excellent cure for diarrhea and to treat various types of inflammation. These phytochemicals have a different mode of action and most of them protect our cells against oxidative damage because they have antioxidant activity (Bhattacharya *et al*., 2012), and others stimulate enzyme activity or interfere with enzyme action (Nakatsuka, 2005).

Table 2.1: Examples of medicinal plants with their major classes of phytochemical (Wangchuk, 2014).

Plant name	Part tested	Tannins	Flavonoids	Alkaloids	Terpenoids	Saponin
Aconitum laciniatum	Tuber	-	-	+++	-	-
Capsella bursa pastoris	Arial	++	++	-	-	-
Fraxinus paxiana	Bark	++	+	-	-	-
Myrsine semiserrata	Fruit	+	+	-	-	-
Gastrococos crispa	Whole	++	-	-	-	-

There are aproximately eight thousand naturally occurring plant phenolics and a large portion of this number are flavonoids (Harborne *et al*., 1993). Phenolics have a wide range of biochemical activities such as antioxidant, antimutagenic, anti-carcinogenic as well as ability to modify gene expression (Swarnalata *et al*., 2016). Phenolics are the biggest group of known phytochemicals that account for majority of the antioxidant activity of plants or plant tissue (Marinova *et al*. 2005). Flavonoids are the largest group of naturally occurring phenolic compounds and are found to have numerous biological activities including antimicrobial, mitochondrial adhesion inhibition, antiulcer, antiarthritic, antiangiogenic, anticancer, and protein kinase inhibition activity (Sulaiman *et al*., 2012).

The flavones and flavonols are the most generally dispersed of the considerable number of phenolics (Peter *et al*., 1999). Flavonoids are especially advantageous, acting as antioxidants and giving protection against cardiovascular sickness, certain types of cancer and age-related degeneration of cell components (Dewick *et al*., 2001). Their polyphenolic nature empowers them to scavenge harmful free radicals such as super oxide and hydroxyl radicals (Arnason *et al*.,

1995). A variety of nutritious plant flavonoids inhibits tumor development in experimental animal models. The bi-flavonoids have the pharmacological impact such as capacity to hinder the release of histamines, the adhesion of blood platelets and the action of lens aldose reductase, to block the inflammatory effects of hepatotoxins, and to act as a heart stimulant (Harborne *et al*., 1988). In light of the solid proof of biological activities of phenolic compounds, the study also focused on determination of total phenolics and flavonoids from *P. grandiflora* tuber extracts.

2.1.2 Methods used for identification

2.1.2.1 Thin-layer chromatography (TLC)

TLC is the simplest, fast, and cost effective procedure that gives the researcher a quick answer as to how many components are in a mixture (Sasidharan *et al*., 2011). TLC can also be utilized to identity compounds in a crude extracts when the R_f of isolated compound is compared with the R_f of a known compound (Sasidharan *et al*., 2011). In order to visualize the isolated bands, the TLC plate is sprayed with phytochemical screening reagents, which cause color changes according to the phytochemicals existing in a plant extracts; or by viewing the plate under the UV light (Anasane and Chaturvedi, 2017). TLC can also be used for confirmation of purity and identity of isolated compounds.

2.1.2.2 Bioautography

Bioautography is one of the methods used to determine presence of a bioactive compound or antibacterial compound within plant extracts (Hamburger and Hostettmann, 1991). Bioautographic methods are used in combination with TLC chromatographic separation methods. Traditionally, the bioautographic method has been used to detect growth inhibition of

microorganisms or anti-microbial components of extracts separated by TLC (Ncube *et al*., 2008). This technique has been regarded as the most effective assay for evaluating anti-microbial compounds (Shahverdi, 2007). This bioautography allows localization of antimicrobial activities of an extract on the TLC plate, and it is also a fast method for searching for new antimicrobial agents (Cosa *et al*., 2006). Bioautography has some advantages when compared to agar diffusion assay as very little amount of sample is needed when compared to the well diffusion assay (Rahalison *et al*., 1991).

2.1.2.3 Fourier-transform infrared spectroscopy (FTIR)

Fourier-transform infrared spectroscopy has been proven to be an important instrument for characterization and identification of compounds or functional groups (chemical bonds) that are present within plants crude extracts (Eberhardt *et al*., 2007; Hazra *et al*., 2007). For identification of the most common plant compounds the FTIR spectrum of an unknown compound can be identified by comparison to a library of known compounds (Sasidharan *et al*., 2011). Some samples need preparation before FTIR analysis. However, for liquid sample, one drop of the sample can easily be placed on the FTIR tool as a thin film between the plates and solid samples can be placed in the FTIR tool and compressed into a thin pellet which can be analyzed (Swapna *et al*., 2015).

2.1.3 Antioxidant activity of plant extracts

The antioxidant activity in plant extracts has been reported to play a major role in reduction of oxidants and reactive oxygen species that can cause various human disorder or diseases (Halliwell *et al*., 1981). This has brought more attention to scientists to find a way to maintain human health either by preventing or treating disorder or disease caused by excess oxidants

(Gulcin *et al*., 2012). Nevertheless, some of the antioxidative mechanism are inherited and some of the biological processes such as the anti-mutagenic, anti-carcinogenic, and anti-aging can also help to reduce oxidant activity (Nunes *et al*., 2012; Gocer *et al*., 2011). Antioxidants work by stabilizing or deactivating free radicals within the human body to prevent them from attacking healthy cells, often before they attack targets in biological cells (Djeridane *et al*., 2006). The antioxidant contents from medicinal plants may also contribute in protecting human and animals from disease (Nunes *et al*., 2012). This medicinal plant extracts with great amount of antioxidant constituents has been reported as an effective therapeutic approach in treatment of hepatic disease (Govind, 2011).

2.1.3 Description of *Pyrenacantha grandiflora* plants used in this study

Pyrenacantha grandiflora Baill belongs to the family Icacinaceae (family of flowering plants). It is characterized by stems with circular whitish lenticels, broadly ovate to almost round leaves that are hairy on both sides (**Figure 2.1)** (Da Silva *et al*., 2004), and is a terrestrial climber tree. A member of the genus that is frequently studied includes *Pyrenacantha staudtii* which was found to possess inhibitory activity on isolated rat uterus (Nworgu *et al*., 2007). *Pyrenacantha grandiflora* is distributed worldwide, but native to Southern Mozambique and South Africa. In South Africa, it is not restricted to a particular locale but it is mostly found in Mpumalanga, KwaZulu-Natal and the Limpopo provinces (Foden and Potter, 2005). In Limpopo, this plant is known by the common names Gwere; Velavhahleka; Mbengelele (Tshivenda); and Velabahleke (Siswati) (Tshisikhawe, 2002). For many years it has been used traditionally in treatment and management of diarrhea.

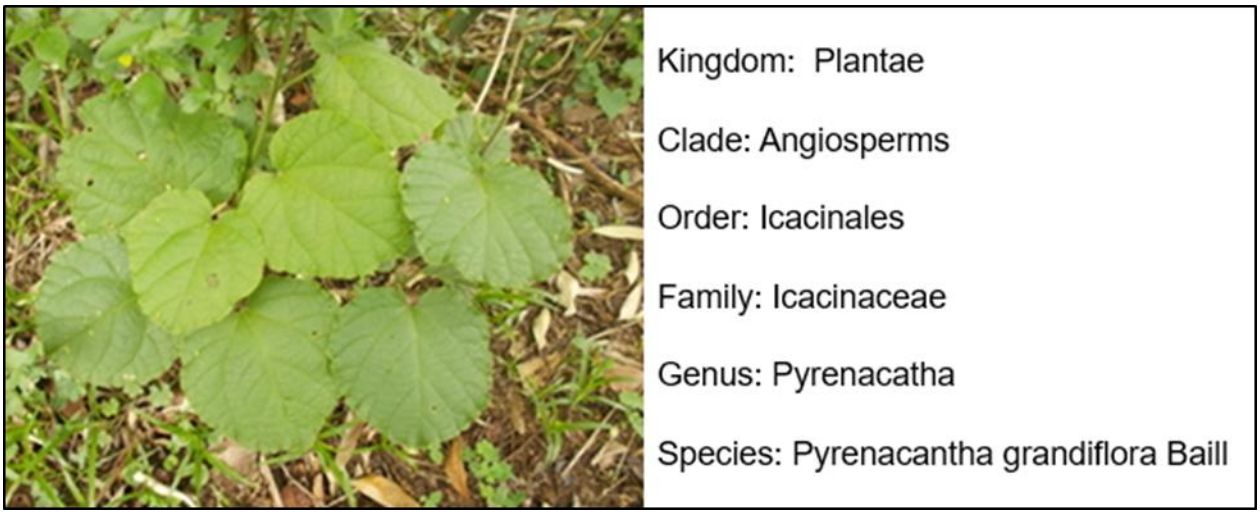


Figure 2.1: Image showing *Pyrenacantha grandiflora* in its natural habitat and its classification

2.2 PROPERTIES OF NANOPARTICLES

Nanoparticles are synthetic materials with dimensions ranging from one to hundred nanometers, some have the same dimension as biological molecules and may be absorbed onto the surface of some large molecules in the host organism (Samberg *et al*., 2012). Over the years, a very wide range of nanoparticles such as silver, gold, zinc, titanium, and magnesium oxide have been engineered from different nanomaterials and have been created via innovative synthetic techniques, yielding a plethora of unique properties and potential applications (Parveen *et al*., 2012).

Nanoparticles are mainly targeted to control drug resistance brought about by genetic mutation of bacterial pathogens. They are able to target biological pathways in a broad spectrum sense. Further advantageous aspects of the use of nanoparticles for their antimicrobial properties include their stability, ensuring a longer shelf-life once manufactured (Weir *et al*., 2008), and their greater degree of resistance to other physical storage and preparation conditions (such as sterilization at high temperatures), to which regular antibiotics display great susceptibility.

Most reported research on nanoparticles has antimicrobial activity based on at least one of the following mechanisms: inhibiting cell wall/membrane synthesis; interrupting energy transduction; production of highly toxic reactive oxygen species, through photocatalysis; and inhibiting the production of enzymes and DNA (Li *et al*., 2008; Weir *et al*., 2008). In terms of the actual antibacterial properties of nanomaterials, metal and metal oxide nanoparticles have received increasing attention, with silver nanoparticles already being incorporated into wound dressings (Rai *et al*., 2015) and polymeric nanoparticles displaying more effective antimicrobial activity against methicillin-resistant Staphylococcus aureus in comparison with general antibiotics (Turos *et al*., 2007). Furthermore, nanoparticles have also been used in the prevention of infectious disease and incorporated into vaccines as colloidal carriers and adjuvants (Kreuter, 1995; Jiang and Koganty, 2003). Some nanoparticles dissolves easily and their effects are the same as the effects from chemically synthesized drugs (Sharma, 2012).

2.2.1 Properties of gold nanoparticles

Gold is a chemical element within the periodic table with an atomic number of 79 and have a thick and delicate dense structure with a yellow-gold color and is separated mine. It has been utilized generally in adornments and decorations from ancient times; and in recent years it has discovered its way into hardware and medicinal application. AuNPs are synthesized in different shape and sizes (**Figure 2.2**). AuNPs are inert and there is mounting proof recommending that they can damage human DNA (Kang *et al*., 2010).

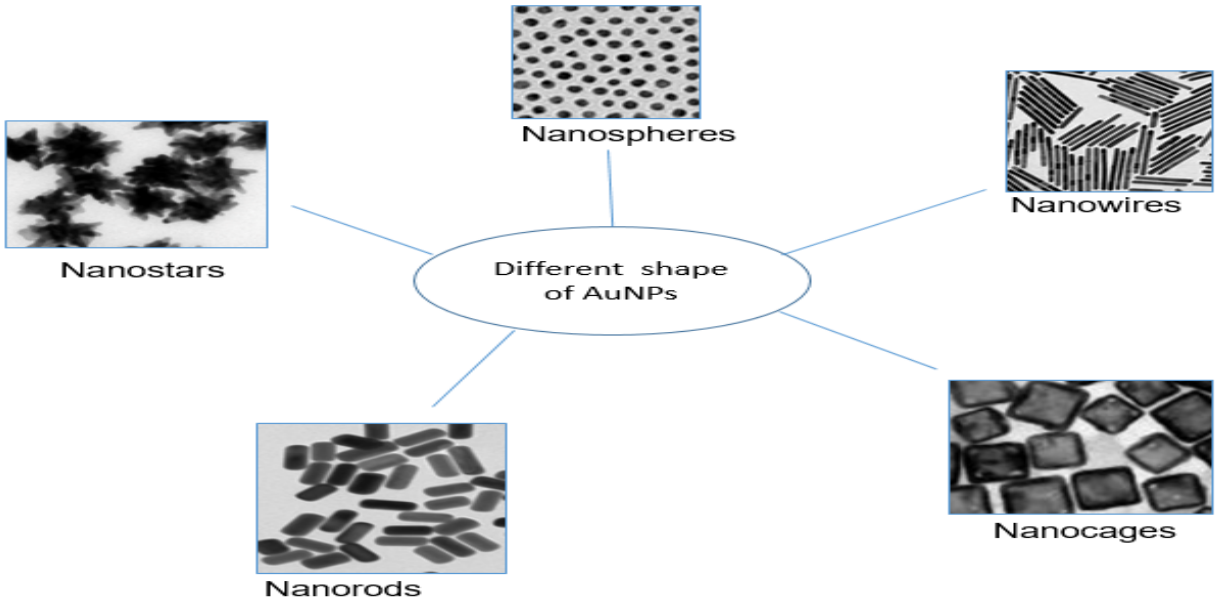


Figure 2.2: Illustration of different shapes of synthesized AuNPs.

Gold nanoparticles have been shown to be toxic to various bacteria (Elsaesser and Howard, 2012). In addition, they cause toxicity by destroying the cell membrane and inhibiting enzymes such as ATPase (Cui *et al*., 2012). Selvaraj and Alagar in 2007 demonstrated that gold nanoparticles show higher antibacterial activity in gram-negative bacteria. Nanoparticles are known to conjugated with the biological system because of their small size, ability to cross the cell membrane, blood and other organs and the ability to spread within the body and solubilize (Elsaesser and Howard, 2012).

2.2.2 Properties of silver nanoparticles

Among nanoparticles, silver nanoparticles have attracted increasing interest due to their distinctive physical, chemical and biological properties when comparing them to their macro-scaled counterparts (Sharma, 2012). Silver nanoparticles have unique physio-chemical properties which includes a high electrical and thermal conductivity, surface-enhanced Raman scattering,

chemical stability, catalytic activity and non-linear optical behavior (Quang *et al*., 2013). Silver nanoparticles have been reported to have broad-spectrum bactericidal and fungicidal activity. Hence, they are well known in a wide range of consumer products, including plastics, soaps, pastes, food, and textiles (Tran *et al*., 2013). Some uses of silver nanoparticles includes use in liquid form such as a colloid (coating and spray) or they can be contained in a bar of soap (solid) (Ahamed , 2010).

2.2.3 Method used for nanoparticles synthesis

Turkevich was the first to demonstrate the synthesis of colloidal gold and silver by means of the citrate reduction technique (Turkevich *et al*., 1951). As this technology improves, the citrate ligand used on these synthesized nanoparticles were replaced with other molecules that have a greater impact on the biomedical application (Schulz *et al*., 2014). The formation of silver nanoparticles requires the reduction of a silver nitrate by an appropriate reducing agent whereas gold nanoparticles requires the reduction of gold salt. This reduction results in nucleation of ionic silver and gold in solution and leads to the production of gold nanoparticles (Turkevich *et al*., 1951).

Thus far, improvement on suitable synthetic techniques for nanoparticle production have been developed due to the physio-chemical properties and also application of nanoparticles (Tiwari *et al*., 2011). Nevertheless, numerous physio-chemical methods for synthesizing metal nanoparticles are hindered by environmental contamination triggered by heavy metals (Singh *et al.*, 2016). Hence, the biological method of nanoparticles syntheses has advantages due to their definite morphology, nontoxicity, and reproducibility in production (Song and Kim, 2009). Hence, various microorganisms such as bacteria, fungi, and yeast, together with plants, have been reported for the synthesis of metallic nanoparticles. For example, *Magnetospirillium*

gryphiswaldense a member of the magnetotactic bacterium family are used in the synthesis of nanoparticles due their excellent metal biosorption (Wang *et al.*, 2013).

2.2.4 Stabilizing agent of nanoparticles

Various stabilizing agents have been tested with gold nanoparticles in direct comparison with citrate, which is the most commonly employed reagent for the synthetic procedure, as it behaves as both a reducing and a stabilizing agent (Tom *et al*., 2004). Other agents that have been used for such stabilizing properties include starch, gum arabic (Kumar *et al*., 2010), sodium borohydride, tannic acid/citrate mixtures and zwitterionic disulfide ligands (Hayat, 1989). More recently, biodegradable polymeric nanoparticles, especially those covered with poly-ethylene glycol (PEG) or any hydrophilic polymer are known to circulate in the biological system for a longer period of time. Furthermore, they have been used as devices to deliver potential medication to their target site, and they also transport DNA in gene therapy (Mohanraj and Chen, 2006). These polymers are non-toxic and also help to reduce nonspecific binding of the nanoparticles.

2.2.5 Methods used to characterize nanoparticles

Different methods are used in the characterization of nanoparticles. Hence, UV-VIS absorbance spectroscopy has been accepted as one of the eminent techniques (Tomaszewska *et al*., 2013). UV-VIS spectroscopy characterizes nanoparticles by using light of various wavelengths to measure the intensities of transmission and incident light (**Figure 2.3**).

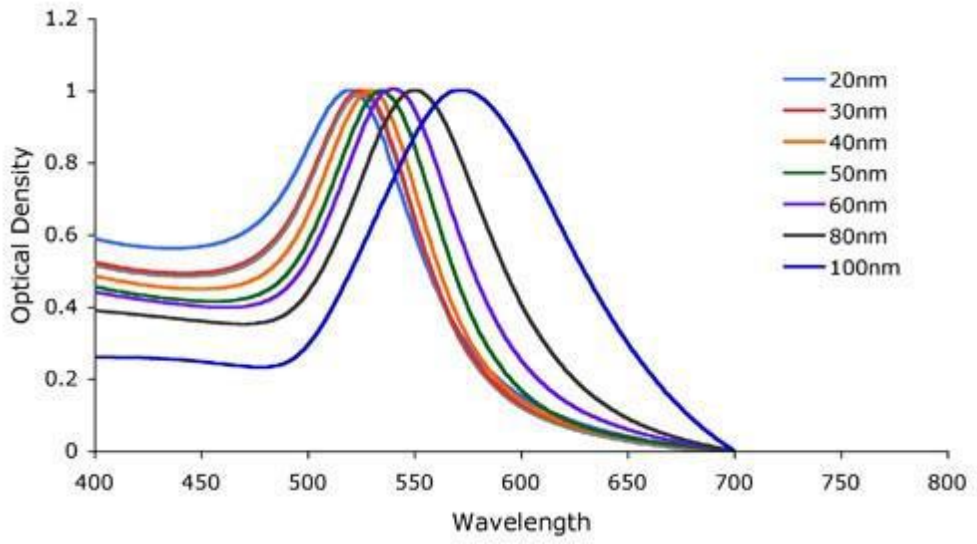


Figure 2.3: Illustration of a typical graph showing UV-VIS absorbance of gold nanospheres having different sizes (Abtahi, 2013).

Thus, if the sample's medium or solvent is changed the peak of absorption value change with the wavelength. TEM is the most common technique used in the characterization of nanoparticles (Mohanraj and Chen, 2006). Moreover, TEM takes the exact image of nanoparticles and allow visualization of the detailed shape, size, and distribution of nanoparticle using different magnification.

2.2.6 Application of nanoparticles

Gold nanoparticles have highly promising potential applications in the field of drug delivery due to advantages such as easy manipulation of particle size and surface characteristics, control and maintenance of the release rate of the medication during transportation, and ability to target specific sites which is accomplished by generating target ligands on the surface of nanoparticles (Sperling *et al*., 2008). AuNPs have a variety of biological applications which can be subdivided

into three categories including labeling, heating, and sensing (Faraji and Wipf, 2009). Their application in the food industry has been highly successful, with antimicrobial activity noted against *E. coli* (Liu *et al*., 2009) and *Staphylococcus aureus* (Uğur *et al*., 2010). They can also be used as a drug itself. Gold nanoparticles are most effective in antimicrobial treatment when used as agents of the hyperthermic effect. This form of antimicrobial application involves the irradiation of gold nanoparticles with light in the near-infrared wavelength range, causing them to heat up and selectively kill bacterial cells which they adhere to (Huang *et al*., 2009). They have been extensively applied in wastewater treatment, as they are non-toxic, stable in an aqueous medium and have a low production cost (Li *et al*., 2008).

2.2.7 Biocompatibility and safety of nanoparticles

AuNPs have inert surface properties which are responsible for their lack of reactivity within biological systems. Various studies have indicated that there is no sign of acute cytotoxicity experienced in the presence of AuNPs (Sperling *et al*., 2008). However, these results vary slightly depending on the cell line used, the stabilizing or coating molecules on the surface of the AuNPs and the method of cytotoxicity testing (Cui *et al*., 2012; Freese *et al*., 2012; Sperling *et al*., 2008; Vijayakumar and Ganesan, 2012). Larger nanoparticles are effectively cleared from the vasculature by phagocytosis. In contrast, very small nanoparticles (between 1 and 20 nm in size) exhibit slow extravasation from the circulatory system and have a much longer retention time (Vijayakumar and Ganesan, 2012). Particles with sizes of the order 30 to 100 nm are able to evade phagocytic mechanisms (Faraji and Wipf, 2009). AuNPs of 13 nm and above are considered non-cytotoxic (Jahnen-Dechent and Simon, 2008), whereas those below 2 nm have been shown to possess active cytotoxic characteristics (Schmid, 2008). Although nanoparticle size ranges seem to contribute greatly to cytotoxic properties on cells, the toxic properties of the

capping agent, sodium citrate/PEG, cannot be ignored in terms of their use in nanoparticle synthesis for biological applications (Freese *et al*., 2012).

2.3 INFECTIOUS PATHOGENIC BACTERIA USED

Infectious microorganisms are usually tested for their degree of resistance to various anti-infective substances in order to prevent the administration of ineffective treatment. Treatment of infections is compromised worldwide by the emergence of bacteria that are resistant to multiple antibiotics. New and reemerging drugs resistant strains used in the present study included *beta*-lactamase producing *Escherichia coli* (ATCC 35218), E*scherichia coli* (ATCC 25922), Methicillin-Resistant *Staphylococcus aureus* (ATCC 25923), Methicillin-Susceptible *Staphylococcus aureus* (ATCC 33594) and *beta*-lactamase producing *Klebsiella pneumonia* (ATCC 700603).

2.3.1 *Beta*-lactamase producing *Escherichia coli*

Escherichia coli are Gram-negative facultative anaerobic bacteria that belongs to the genus *Escherichia* (Tenaillon *et al*., 2010). They are commonly found in the lower intestine of warm-blooded organisms and are part of the normal flora (Roberts, 2015). *Escherichia coli* is used as an important indicator of contamination in the ecosystem, water, soil and food (Edberg *et al*., 2000). However, *E. coli* causes a wide range of diseases and infection such as urinary tract infection, wounds, beadsores, gastrointestinal infections (Falagas *et al*., 2010). In 2002, Bell et al. reported the prevalence of extended spectrum β-lactamase (ESBL)-producing clinical isolates in the Asia-Pacific region and South Africa. Resistance of *E. coli* has been observed for many medicines. For example, treatment of urinary tract infection with fluoroquinolone is very widespread (Ismail *et al*., 2018). Hence, there are various countries in different parts of the world

where this treatment is now ineffective. Multidrug resistance of Enterobacteriaceae including *E. coli* produce extended spectrum *β*-lactamases such as CTX-M (Shaikh *et al*., 2015).

2.3.2 Beta-lactamase *Klebsiella pneumonia*

Klebsiella pneumonia is also a Gram-negative facultative anaerobic bacteria that belongs to the family Enterobacteriaceae that causes destructive changes in human and animal lungs if it is aspirated into the lungs (Grimont and Grimont, 2015). Although found in the normal flora of the mouth, skin, and intestines, infections are mostly observed in people with weak immune systems. In Asia *K. pneumonia* has been reported to cause community-acquired pneumonia which can result in death (Yan *et al*., 2015) whilst in South Africa, Tau et al. (2012) report that more than half of hospitalized patients has extended spectrum *beta*-lactamase producing *K. pneumonia*.

2.3.3 Methicillin-resistant *Staphylococcus aureus*

Staphylococcus aureus is a facultative anaerobic Gram-positive bacterium that is usually found in the skin and respiratory tract of humans (Grimont and Grimont, 2015). It is also one of the main causes of community acquired and hospital acquired infection that are causing a variety of symptoms such as food poisoning and skin lesions (Masalha *et al*., 2001). *Staphylococcus aureus* have been reported as penicillin resistant, and this resistance is enhanced by the production of penicillinase (a form of *β*-lactamase) (Ibuka *et al*., 2003). Hence, this enzyme can cut the *β*-lactam ring of the penicillin molecule, resulting in an ineffective antibiotic. Nevertheless, some penicillinase-resistant *β*-lactam antibiotics, such as methicillin, nafcillin, oxacillin, cloxacillin, dicloxacillin, and flucloxacillin, are able to resist degradation by staphylococcal penicillinase (Jalalpour, 2012). In South Africa, Van Boeckel *et al*. (2014) reported that more than 50% of all

hospitalized patient from 2000-2010 acquired methicillin resistance *S. aureus* (MRSA) and some were resistant to *β*-lactam antibiotics.

2.4 ANTIBIOTICS USED IN THIS STUDY

2.4.1 Penicillin

Penicillin is a group of antibiotics that was among the first medication used in treatment of bacterial infections by killing the bacteria and preventing their growth (Hanlon, 2007). There are several different kinds of penicillin which includes Penicillin G, procaine penicillin and penicillin V, and one kind of penicillin usually cannot be replaced by another since they have different routes of administration (Thaden *et al*., 2015). To enhance the activity of penicillin, it can be prescribed together with other antibacterial medicines (Silve and Bostian, 1993) because it prevents the final cross-linking step, or transpeptidation, in the assembly of bacterial macromolecules. This results in a very fragile cell wall that bursts due to osmosis,thereby killing the bacterium (Koch,1990).

2.4.2 Ampicillin

Antibiotic resistance have been reported in many bacterial strains, however, the resistance that is most commonly used in research and industry is ampicillin resistance (Blair *et al*., 2015). It is mostly used as a selectable marker in bacterial transformation in order to allows for the scientist to select only the bacterial cells that contain the gene that allow the bacterium to be resistant (Lewis and Shan, 2017). Ampicillin have also been used in treatment of respiratory tract infections and urinary tract infections (Ravina, 2011). Ampicillin falls under *beta*-lactam producing antibiotics

and it is in the same group as penicillin. Yet, bacteria are becoming resistant to antibiotics that used to work on them (Elmahdi *et al*., 2016).

2.4.3 Vancomycin

Vancomycin has been the foundation of therapy for serious methicillin-resistant *Staphylococcus aureus* (MRSA) infections since the early 1980s, when MRSA emerged as a significant nosocomial pathogen (Leeb and Howden, 2015). However, many scientists believed that the efficacy of vancomycin against MRSA is inferior compared to antistaphylococcal *beta*-lactams against methicillin-susceptible *S. aureus* (MSSA) infections (Sakoulas *et al*., 2004). The origin of this belief comes partially from *in vitro* data that demonstrate slower bactericidal activity of vancomycin compared to the results seen with antistaphylococcal *beta*-lactams against *S. aureus* as well as data suggesting a slow clinical response (Sakoulas *et al.,* 2004).

2.5 *REFERENCES*

Abbasi, E., Milani, M., Fekri Aval, S., Kouhi, M., Akbarzadeh, A., Tayefi Nasrabadi, H., Nikasa, P., Joo, S.W., Hanifehpour, Y., Nejati-Koshki, K. and Samie, M., (2016). Silver nanoparticles: synthesis methods, bio-applications and properties. *Critical reviews in microbiology*, 42(2), pp.173-180.

Abtahi, S.M.H., (2013). Synthesis and characterization of metallic nanoparticles with photoactivated surface chemistries (Doctoral dissertation, Virginia Tech).

Ahmad, M. Z., Akhter, S., Rahman, Z., Akhter, S., Anwar, M., Mallik, N., & Ahmad, F. J. (2013). Nanometric gold in cancer nanotechnology: current status and future prospect. *Journal of Pharmacy and Pharmacology*, 65(5), pp.634-651.

Aiyegoro, O.A., Akinpelu, D.A., Afolayan, A.J. and Okoh, A.I., (2008). Antibacterial activities of crude stem bark extracts of *Distemonanthus benthamianus* Baill. *Saudi Journal of Biological Sciences*. 8(2), pp.356-361.

Alachaher, F.Z., Dali, S., Dida, N. and Krouf, D., (2018). Comparison of phytochemical and antioxidant properties of extracts from flaxseed (*Linum usitatissimum*) using different solvents. *International Food Research Journal*, 25(1).

Almeida, J.P.M., Figueroa, E.R., and Drezek, R.A., (2014). Gold nanoparticle mediated cancer immunotherapy. *Nanomedicine* 10, 503–514.doi: 10.1016/j.nano.2013.09.011

Anasane, P. and Chaturvedi, A., (2017). Pharmacognostic studies and qualitative analysis for phytosterols of the leaves of *Bombax Ceiba* Linn. *International Journal Of Pharmaceutical Sciences And Research*, 8(10), pp.4402-4407.

Arnason, J.T., Mata, R., and Romeo, J.T., (1995). Recent trends in Phytochemistry, Plenum Press New York and London.

Bell, J.M., Turnidge, J.D., Gales, A.C., Pfaller, M.A. and Jones, R.N., (2002). Prevalence of extended spectrum *β*-lactamase (ESBL)-producing clinical isolates in the Asia-Pacific region and South Africa: regional results from SENTRY Antimicrobial Surveillance Program (1998–99). *Diagnostic microbiology and infectious disease*, 42(3), pp.193-198.

Bhattacharya, D., Biswarup, S., Mukherjee, A., Ranjan, S.C., Parimal, K., (2012) Gold Nanoparticles Conjugated Antibiotics: Stability and Functional Evaluation. *Nanoscience and Nanotechnology*. 2(2): 14-21 DOI: 10.5923/j.nn.20120202.04.

Bhattacharya, I., (2012). Evaluation of antioxidant capacityand phenol content of five different vegetables from kolkata. Barrackpore Rastraguru Surendranath College (Masters dissertation).

Blair, J.M., Webber, M.A., Baylay, A.J., Ogbolu, D.O. and Piddock, L.J., (2015). Molecular mechanisms of antibiotic resistance. *Nature Reviews Microbiology*, 13(1), p.42.

Boucher, H.W., Bakken, J.S. and Murray, B.E., (2016). The United Nations and the Urgent Need for Coordinated Global Action in the Fight against Antimicrobial Resistance Coordinated Global Action to Fight against Antimicrobial Resistance. *Annals of Internal Medicine*, 165(11), pp.812-813.

Brown, A.N., Smith, K., Samuels, T.A., Lu, J., Obare, S.O. and Scott, M.E. (2012). Nanoparticles Functionalized with Ampicillin Destroy Multiple-Antibiotic-Resistant Isolates of *Pseudomonas aeruginosa* and *Enterobacter aerogenes* and Methicillin-Resistant *Staphylococcus aureus*. *Applied and environmental microbiology*, 78, 2768-2774.

Clatworthy, A.E., Pierson, E. and Hung, D.T., (2007). Targeting virulence: a new paradigm for antimicrobial therapy. *Nature chemical biology*, 3(9), p.541.

Cos, P., Vlietinck, A.J., Berghe, D.V. and Maes, L., (2006). Anti-infective potential of natural products: how to develop a stronger *in vitro* 'proof-of-concept'. *Journal of ethnopharmacology*, 106(3), pp.290-302.

Cui, Y., Zhao, Y., Tian, Y., Zhang, W., Lü, X. & Jiang, X. (2012). The molecular mechanism of action of bactericidal gold nanoparticles on *Escherichia coli*. *Biomaterials*, 33, 2327-2333

Da Silva, M.C., Izidine, S. and Amude, A.B., (2004). A preliminary checklist of the vascular plants of Mozambique. Southern African Botanical Diversity Network Report No. 30 Sabonet, Pretoria Page 76.

Devaraj, P., Kumari, P., Aarti, C. and Renganathan, A., (2013). Synthesis and characterization of silver nanoparticles using cannonball leaves and their cytotoxic activity against MCF-7 cell line. *Journal of nanotechnology*.

Dewick, P.M., (2001). Medicinal natural products A Biosynthetic approach, Second Edition, John Wiley & Sons, Ltd, Chichester, UK. doi: 10.1002/0470846275.

Djeridane, A., Yousfi, M., Nadjemi, B., Boutassouna, D., Stocker, P. and Vidal, N., (2006). Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds. *Food chemistry*, 97(4), pp.654-660.

Drbohlavova, J., Chomoucka, J., Adam, V., Ryvolova, M., Eckschlager, T., Hubalek, J. and Kizek, R., (2013). Nanocarriers for anticancer drugs-new trends in nanomedicine. *Current drug metabolism*, 14(5), pp.547-564.

Duraipandiyan, V., Ayyanar, M. and S. Ignacimuthu., (2006). Antimicrobial activity of some ethnomedicinal plants used by Palyar tribe from Tamil Nadu, India. *BMC Complement. Alternative Med*icine 6: 35–41.

Eberhardt, T.L., Li, X., Shupe, T.F. and Hse, C.Y., (2007). Chinese Tallow Tree (*Sapium Sebiferum*) utilization: Characterization of extractives and cell-wall chemistry. *Wood and Fiber Science*, 39(2), pp.319-324.

Edberg, S.C.L., Rice, E.W., Karlin, R.J. and Allen, M.J., (2000). *Escherichia coli*: the best biological drinking water indicator for public health protection. *Journal of Applied Microbiology*, 88(S1).

Elmahdi, S., DaSilva, L.V. and Parveen, S., (2016). Antibiotic resistance of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in various countries: a review. *Food microbiology*, 57, pp.128-134.

Eloff, J.N., Famakin, J.O., and Katerere, D.R., (2005). *Combretum woodii* (Combretaceae) leaf extracts have high activity against Gram-negative and Gram-positive bacteria, *African Journal of Biotechnology*, 4, pp. 1161-1166.

Elsaesser, A. and Howard, C.V., (2012). Toxicology of nanoparticles. *Advanced drug delivery reviews*, 64(2), pp.129-137.

Falagas, M.E., Kastoris, A.C., Kapaskelis, A.M. and Karageorgopoulos, D.E., (2010). Fosfomycin for the treatment of multidrug-resistant, including extended-spectrum *β*-lactamase producing, Enterobacteriaceae infections: a systematic review. *The Lancet infectious diseases*, 10(1), pp.43-50.

Faraji, A.H. and Wipf, P., (2009). Nanoparticles in cellular drug delivery. *Bioorganic & medicinal chemistry*, 17, 2950-2962.

Foden, W. and Potter, L., (2005). *Pyrenacantha grandiflora* Baill. National Assessment: Red List of South African Plants version 2017.1. Accessed on 2017/07/06.

Freese, C., Uboldi, C., Gibson, M.I., Unger, R.E., Weksler, B.B., Romero, I.A., Couraud, P.O. and Kirkpatrick, C.J., (2012). Uptake and cytotoxicity of citrate-coated gold nanospheres: Comparative studies on human endothelial and epithelial cells. *Particle and Fibre Toxicology*, 9, 1-11.

Giljohann, D.A., Seferos, D.S., Daniel, W.L., Massich, M.D., Patel, P.C. and Mirkin, C.A., (2010). Gold nanoparticles for biology and medicine. Angewandte Chemie International Edition, 49(19), pp.3280-3294.

Gindy, M.E. and Prud'homme, R.K., (2009). Multifunctional nanoparticles for imaging, delivery and targeting in cancer therapy. *Expert opinion on drug delivery*, 6(8), pp.865-878.

Göçer, H. and Gülçin, İ., (2011). Caffeic acid phenethyl ester (CAPE): correlation of structure and antioxidant properties. *International Journal of Food Sciences and Nutrition*, 62(8), pp.821-825.

Govind, P., (2011). Medicinal plants against liver diseases. *International Journal of Production Research*, 2, pp.115-121.

Taylor, P. W., Stapleton, P. D. & Paul Luzio, J. (2002). New ways to treat bacterial infections. *Drug Discovery* Today, 7, 1086-1091.

Grimont, P.A. and Grimont, F., (2015). *Klebsiella*. Bergey's Manual of Systematics of Archaea and Bacteria, pp.1-26.

Gülçin, I., (2012). Antioxidant activity of food constituents: an overview. *Archives of toxicology*, 86(3), pp.345-391.

Gundidza, M., Gweru, N., Mmbengwa, V., Ramalivhana, N.J., Magwa, Z. and Samie, A., (2009). Phytoconstituents and biological activities of essential Oil from *Rhus lancea* L. F. *African Journal of Biotechnology* 7 (16): 2787-2789.

Halliwell, B., and Gutteridge, J.M.C., (1981). Formation of thiobarbituric acid reactive substances from deoxyribose in the presence of iron salts: the role of superoxide and hydroxyl radicals. *FEBS Letters.* 1981, 128:347–352.

Hamburger, M., and Hostettmann, K., (1991). Bioactivity in plants: the link between phytochemistry and medicine. *Phytochemistry*, 30(12), pp.3864-3874.

Hanlon, G.W., (2007). Bacteriophages: an appraisal of their role in the treatment of bacterial infections. *International journal of antimicrobial agents*, 30(2), pp.118-128.

Harborne, J.B., (2013). The flavonoids: advances in research since 1980. Springer. Harman, D., (1988). Free radical theory of aging: current status. Lipofuscin-1987: State of the art, 1, pp.3-21.

Hayat, M.A., (2012). Colloidal gold: principles, methods, and applications. *Elsevier*.

Hazra, K.M., Roy, R.N., Sen, S.K. and Laskar, S., (2007). Isolation of antibacterial pentahydroxy flavones from the seeds of *Mimusops elengi* Linn. *African journal of Biotechnology*, 6(12).

Huang, W.C., Tsai, P.J. and Chen, Y.C., (2009). Multifunctional Fe3O4@ Au nanoeggs as photothermal agents for selective killing of nosocomial and antibiotic-resistant bacteria. Small, 5(1), pp.51-56.

Huh, A.J. and Kwon, Y.J. (2011). Nanoantibiotics‖: A new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era. *Journal of Controlled Release*, 156, 128-145.

Ibuka, A.S., Ishii, Y., Galleni, M., Ishiguro, M., Yamaguchi, K., Frère, J.M., Matsuzawa, H. and Sakai, H., (2003). Crystal structure of extended-spectrum *β*-lactamase Toho-1: insights into the molecular mechanism for catalytic reaction and substrate specificity expansion. *Biochemistry*, 42(36), pp.10634-10643.

Ismail, M.D., Ali, I., Hatt, S., Salzman, E.A., Cronenwett, A.W., Marrs, C.F., Rickard, A.H. and Foxman, B., (2018). Association of *Escherichia coli* ST131 lineage with risk of urinary tract infection recurrence among young women. *Journal of global antimicrobial resistance*, 13, pp.81-84.

Jahnen-Dechent, W. and Simon, U. (2008). Function follows form: shape complementarity and nanoparticle toxicity. *Nanomedicine* (London, England), 3, 601.

Jalalpour, S., (2012). Food borne diseases bacteria; frequency antibiotic resistance bacteria in Iranian foods. *African Journal of Microbiology Research*, 6(4), pp.719-723.

Jason, N., Payne, H.K., Waghwani, M.G., Connor, W.H., Tockstein, S., Moolani, H., Chavda, F., Badwaik, V., Matthew, B.L. and Rajalingam D., (2016). Novel Synthesis of Kanamycin Conjugated Gold Nanoparticles with Potent Antibacterial Activity. *Forntier in Microbiology*. doi: 10.3389/fmicb.2016.00607

Jiang, Z.H. and Koganty, R., (2003). Synthetic vaccines: the role of adjuvants in immune targeting. *Current medicinal chemistry*, 10, 1423-1439.

Kappers, I.F., Aharoni, A., van Herpen, T.W., Luckerhoff, L.L., Dicke, M., et al. (2005) Genetic engineering of terpenoid metabolism attracts bodyguards to Arabidopsis. *Science* 309: 2070-2072.

Kimling, J., Maier, M., Okenve, B., Kotaidis, V., Ballot, H. & Plech, A. (2006). Turkevich method for gold nanoparticle synthesis revisited. *The Journal of Physical Chemistry* B, 110, 15700-15707.

Koch, A.L., (1990). Growth and form of the bacterial cell wall. *American Scientist*, 78(4), pp.327-341.

Kreuter, J. (1995). Nanoparticles as adjuvants for vaccines. Vaccine Design. *Springer*.

Krishnaiah, D., Sarbatly, R. and Bono, A., (2007). Phytochemical antioxidants for health and medicine a move towards nature. *Biotechnology and Molecular Biology Reviews*, 2(4), pp.97-104.

Kumar, S.A., Chang, Y.T., Wang, S.F. and Lu, H.C., (2010). Synthetic antibacterial agent assisted synthesis of gold nanoparticles: Characterization and application studies. *Journal of Physics and Chemistry of Solids*, 71, 1484-1490.

Lee, J.Y. and Howden, B.P., (2015). Vancomycin in the treatment of methicillin-resistant *Staphylococcus aureus*–a clinician's guide to the science informing current practice. *Expert review of anti-infective therapy*, 13(7), pp.855-869.

Lewis, K. and Shan, Y., (2017). Why tolerance invites resistance. *Science*, 355(6327), pp.796-796.

Li, L., Liu, X., Fu, C., Tan, L. and Liu, H., (2015). Biosynthesis of fluorescent gold nanoclusters for *in vitro* and in vivo tumor imaging. *Optics Communications*, 355, pp.567-574.

Liao, H. and Hafner, J.H., (2005). Gold nanorod bioconjugates. *Chemistry of Materials*, 17(18), pp.4636-4641.

Liu, Y., He, L., Mustapha, A., Li, H., Hu, Z. and Lin, M. (2009). Antibacterial activities of zinc oxide nanoparticles against *Escherichia coli* O157: H7. *Journal of Applied Microbiology*, 107, 1193-1201.

Livermore, D.M., (2003). Bacterial resistance: origins, epidemiology, and impact. *Clinical Infectious Diseases*, 36, S11-S23.

Mahato, S.B., Sen, S., (1997). Advances in triterpenoid research, 1990-1994. *Phytochemistry* 44: 1185-1236.

Malik, F., Hussain, D., Dil A.S., Hannan, A., Gilani. A.H., (2005). WHO Global Atlas of Traditional, Complementary and Alternative Medicine, Islamic republic of Pakistan. In: Ong, C.K., G. Bodeker, C. Grundy, G. Burford and K. Shein. (Eds.). (Map Volume). World Health Organization. Geneva. pp. 165–169 (Chapter22).

Marinova, D., Ribarova, F. and Atanassova, M., (2005). Total phenolics and total flavonoids in Bulgarian fruits and vegetables. *Journal of the university of chemical technology and metallurgy*, 40(3), pp.255-260.

Masalha, M., Borovok, I., Schreiber, R., Aharonowitz, Y. and Cohen, G., (2001). Analysis of Transcription of the *Staphylococcus aureus* Aerobic Class Ib and Anaerobic Class III Ribonucleotide Reductase Genes in Response to Oxygen. *Journal of bacteriology*, 183(24), pp.7260-7272.

Mohanraj, V.J. and Chen, Y., (2006). Nanoparticles – A Review. *Tropical Journal of Pharmaceutical Research*. 5 (1): 561-573.

Mustafa, G., Rawaba A., Asia A., Sumaira, S. and Amer J., (2017), Bioactive Compounds from Medicinal Plants and Their Importance in Drug Discovery in Pakistan. *Matrix Science Pharmaceutical* (MSP) 1) 17-26. 2455-0986.

Nakatsuka, T., Nishihara, M., Mishiba, K., Yamamura, S. (2005).Temporal expression of flavonoid biosynthesis-related genes regulates flower pigmentation in gentian plants. *Plant Science*, 168 pp. 1309-1318.

Ncube, N.S., Afolayan, A.J. and Okoh, A.I., (2008). Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. *African journal of biotechnology*, 7(12).

Nostro, A., Germano, M.P., D'angelo, V., Marino, A. and Cannatelli, M.A., (2000). Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. *Letters in applied microbiology*, 30(5), pp.379-384.

Nunes, X.P., Silva, F.S., Almeida, J.R.G.D.S., de Lima, J.T., de Araújo Ribeiro, L.A., Júnior, L.J.Q. and Barbosa Filho, J.M., (2012). Biological oxidations and antioxidant activity of natural products.

In Phytochemicals as nutraceuticals-Global Approaches to Their Role in Nutrition and Health. *InTech*.

Nworgu, Z.A., Falodun, A. and Usifoh, C.O., (2007). Inhibitory Effect Of 3-Carbomethoxypyridine and 3-Carbobutoxypyridine On Isolated Rat Uterus. *Acta poloniae pharmaceutical*, 64(2), pp.179-182.

O'Bryan, C.A., Crandall, P.G. and Ricke, S.C., (2018). Antimicrobial Resistance in Foodborne Pathogens. *In Food and Feed Safety Systems and Analysis* (pp. 99-115).

Osawa, T., Namiki, M. and Kawakishi, S., 1990. Role of dietary antioxidants in protection against oxidative damage. In Antimutagenesis and anticarcinogenesis mechanisms II (pp. 139-153). *Springer*, Boston, MA.

Parveen, S., Misra, R. & Sahoo, S.K. (2012). Nanoparticles: a boon to drug delivery, therapeutics, diagnostics and imaging. *Nanomedicine: Nanotechnology, Biology and Medicine*, 8, 147-166.

Patil, S.T., Zhang, L., Martenyi, F., Lowe, S.L., Jackson, K.A., Andreev, B.V., Avedisova, A.S., Bardenstein, L.M., Gurovich, I.Y., Morozova, M.A. and Mosolov, S.N., (2007). Activation of mGlu2/3 receptors as a new approach to treat schizophrenia: a randomized Phase 2 clinical trial. *Nature medicine*, 13(9), p.1102.

Pereira, L., Mehboob, F., Stams, A.J., Mota, M.M., Rijnaarts, H.H. and Alves, M.M., (2015). Metallic nanoparticles: microbial synthesis and unique properties for biotechnological applications, bioavailability and biotransformation. *Critical reviews in biotechnology*, 35(1), pp.114-128.

Peter. B., Kaufman, L. J., Warber, C.S., James, A., Harry, D.L., (1999). Natural products from Plants, CRC Press London.

Quang, D.V., Lee, J.E., Kim, J.K., Kim, Y.N., Shao, G.N. and Kim, H.T., (2013). A gentle method to graft thiol-functional groups onto silica gel for adsorption of silver ions and immobilization of silver nanoparticles. *Powder technology*, 235, pp.221-227.

Rahalison, L.L., Hamburger, M., Hostettmann, K., Monod, M. and Frenk, E., 1991. A bioautographic agar overlay method for the detection of antifungal compounds from higher plants. *Phytochemical analysis*, 2(5), pp.199-203.

Rajeh, M.A.B., Zuraini, Z., Sasidharan, S., Latha, L.Y. and Amutha, S., (2010). Assessment of *Euphorbia hirta* L. leaf, flower, stem and root extracts for their antibacterial and antifungal activity and brine shrimp lethality. *Molecules*. 15:6008–18.

Ramalivhana, N.J., (2010). Molecular characterization of *Aeromonas hydrophila* and antimicrobial activities of selected medicinal plants against pathogenic isolates from water and stool samples in the era of HIV/AIDS in Limpopo Province, South Africa (Doctoral dissertation).

Rasool Hassan Bassam Abdul, (2012).Medicinal plants (Importants and uses). *Pharmaceutical Analytica Acta* 3: e139. doi:10.4172/2153-2435.1000e139

Raviña, E., (2011). The evolution of drug discovery: from traditional medicines to modern drugs. John Wiley & Sons.

Roberts, R.M., (2015). Prevalence of Trichobilharzia and *Escherichia Coli* in Missouri Lake Systems (Doctoral dissertation).

Sakoulas, G., Moise-Broder, P.A., Schentag, J., Forrest, A., Moellering, R.C. and Eliopoulos, G.M., (2004). Relationship of MIC and bactericidal activity to efficacy of vancomycin for treatment of methicillin-resistant *Staphylococcus aureus* bacteremia. *Journal of clinical microbiology*, 42(6), pp.2398-2402.

Samberg, M.E., and Monteiro-Riviere, N.A., (2012). In vitro and in vivo toxicity of silver nanoparticles. In *Encyclopedia of nanotechnology* (pp. 1069-1077). *Springer* Netherlands.

Samie, A., Ramalivhana, J., Igumbor, E.O. and Obi, C.L., (2007). Prevalence, Haemolytic and haemagglutination activities and antibiotic susceptibility profiles of Campylobacter spp. isolated from diarrhoeal stools in Vhembe district, South Africa. Journal of Health Population and Nutrition 25(4): 406-413.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K.M. and Latha, L.Y., (2011). Extraction, isolation and characterization of bioactive compounds from plants' extracts. *African Journal of Traditional, Complementary and Alternative Medicines*, 8(1).

Savoia, D., (2012). Plant-derived antimicrobial compounds: alternatives to antibiotics. p. 979–90.

Schmid, G. (2008). The relevance of shape and size of Au55 clusters. *Chemical Society Reviews*, 37, 1909-1930.

Schulz, F., Homolka, T., Bastús, N.G., Puntes, V., Weller, H. and Vossmeyer, T., (2014). Little adjustments significantly improve the Turkevich synthesis of gold nanoparticles. *Langmuir*, 30(35), pp.10779-10784.

Selvaraj, V. and Alagar, M., (2007). Pt and Pt–Ru nanoparticles decorated polypyrrole/multiwalled carbon nanotubes and their catalytic activity towards methanol oxidation. *Electrochemistry Communications,* 9(5), pp.1145-1153.

Shah, M., Badwaik, V., Kherde, Y., Waghwani, H.K., Modi, T., Aguilar, Z.P., (2014).Goldnanoparticles: various methods of synthesis and antibacterial applications. *Frontier Biosciences*. 19:1320. doi: 10.2741/4284.

Shahverdi, A.R., Abdolpour, F., Monsef-Esfahani, H.R. and Farsam, H., (2007). A TLC bioautographic assay for the detection of nitrofurantoin resistance reversal compound. *Journal of Chromatography B*, 850(1-2), pp.528-530.

Shaikh, S., Fatima, J., Shakil, S., Rizvi, S.M.D. and Kamal, M.A., (2015). Antibiotic resistance and extended spectrum *beta*-lactamases: types, epidemiology and treatment. *Saudi journal of biological sciences*, 22(1), pp.90-101.

Sharma, R., (2012). Effect of Nanoparticles on the Human Health and Environment. *Journal of Pure Applied and Industrial Physics*. Vol, *2*(3A), pp.286-402.

Silver, L.L. and Bostian, K.A., (1993). Discovery and development of new antibiotics: the problem of antibiotic resistance. *Antimicrobial agents and chemotherapy*, 37(3), p.377.

Singh, P., Kim, Y.J., Zhang, D. and Yang, D.C., (2016). Biological synthesis of nanoparticles from plants and microorganisms. *Trends in biotechnology*, 34(7), pp.588-599.

Singleton, V.L. and Rossi, J.A., (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American journal of Enology and Viticulture*, 16(3), pp.144-158.

Song, J.Y. and Kim, B.S., (2009). Rapid biological synthesis of silver nanoparticles using plant leaf extracts. *Bioprocess and biosystems engineering*, 32(1), p.79.

Sperling, R.A., Gil, P.R., Zhang, F., Zanella, M. & Parak, W.J. (2008). Biological applications of gold nanoparticles. *Chemical Society Reviews*, 37, 1896-1908.

Sulaiman, C.T., Sadashiva, C.T., George, S., Goplakrishnan, V.K. and Balachandran, I., (2013). Chromatographic studies and in vitro screening for acetyl cholinesterase inhibition and antioxidant activity of three Acacia Species from South India. *Analytical Chemistry Letters*, 3(2), pp.111-118.

Sulaiman, C.T. and Balachandran, I., (2012). Total phenolics and total flavonoids in selected Indian medicinal plants*. Indian journal of pharmaceutical sciences*, 74(3), p.258.

Swapna, G., Jyothirmai, T., Lavanya, V., Swapnakumari, S. and Sri Lakshmi, P.A., (2015). Extraction, and characterization of bioactive compounds from plant extracts: A review. European *Journal of Pharmaceutical Science and Research*, 2, pp.1-6.

Swarnalata, N., Sharma, S., Tiwari, A. and Latha, S., (2016). Phytochemical Constituents, Total Flavonoid and Phenolic Content of *Allium hookeri* Thw. Enum. Leaf Extracts and their Antioxidant Potential. *Journal of Academia and Industrial Research* (JAIR), 5(4), p.61.

Tau, N.P., Smith, A.M., Sooka, A. and Keddy, K.H., (2012). for the Group for Enteric, Respiratory, and Meningeal Disease Surveillance in South Africa (GERMS-SA). Molecular characterization of extended-spectrum *beta*-lactamase-producing Shigella isolates from humans in South Africa, 2003-2009. *Journal of Medical Microbiology*, 61(Pt 1), pp.162-164.

Tenaillon, O., Skurnik, D., Picard, B. and Denamur, E., (2010). The population genetics of commensal *Escherichia coli*. *Nature Reviews Microbiology*, 8(3), p.207.

Thaden, J.T., Ericson, J.E., Cross, H., Bergin, S.P., Messina, J.A., Fowler Jr, V.G., Benjamin Jr, D.K., Clark, R.H., Hornik, C.P. and Smith, P.B., (2015). Survival benefit of empirical therapy for *Staphylococcus aureus* blood stream infections in infants. *The Pediatric infectious disease journal*, 34(11), p.1175.

Tillotson, G.S., and Theriault, N., (2013). New and alternative approaches to tackling antibiotic resistance. *F1000 Prime Reports*. 5:51. doi: 10.12703/P5-51.

Tiwari, P.M., Vig, K., Dennis, V.A. and Singh, S.R., (2011). Functionalized gold nanoparticles and their biomedical applications. *Nanomaterials*, 1(1), pp.31-63.

Tomaszewska, E., Soliwoda, K., Kadziola, K., Tkacz-Szczesna, B., Celichowski, G., Cichomski, M., Szmaja, W. and Grobelny, J., (2013). Detection limits of DLS and UV-Vis spectroscopy in characterization of polydisperse nanoparticles colloids. *Journal of Nanomaterials*, *2013*, p.60.

Tran, Q.H. and Le, A.T., (2013). Silver nanoparticles: synthesis, properties, toxicology, applications and perspectives. *Advances in Natural Sciences: Nanoscience and Nanotechnology*, 4(3), p.033001.

Tshisikhawe, M.P., 2002. Trade of indigenous plants in the Northern Province, Venda region: Their ethnobotanical importance and sustainable use (Doctoral dissertation, M. Sc. dissertation. University of Venda. Thohoyandou, RSA.

Turkevich, J., Stevenson, P.C. and Hillier, J. (1951). A study of the nucleation and growth processes in the synthesis of colloidal gold. *Discussions of the Faraday Society*, 1951. 11: p. 55-75.

Turos, E., Reddy, G.S., Greenhalgh, K., Ramaraju, P., Abeylath, S.C., Jang, S., Dickey, S., Lim D.V. (2007). Penicillin-bound polyacrylate nanoparticles: restoring the activity of *β*-lactam antibiotics against MRSA. *Bioorganic Medical Chemistry Letters*., 17 pp. 3468-3472.

Uğur, Ş.S., Sarıışık, M., Aktaş, A.H., Uçar, M.Ç. and Erden, E. (2010). Modifying of cotton fabric surface with nano-ZnO multilayer films by layer-by-layer deposition method. *Nanoscale research letters*, 5, pp.1204-1210.

Upadhyay, A., Upadhyaya, I., Kollanoor-Johny, A. and Venkitanarayanan, K., (2014). Combating pathogenic microorganisms using plant-derived antimicrobials: a minireview of the mechanistic basis. *BioMed research international*, 2014.

Van Boeckel, T.P., Gandra, S., Ashok, A., Caudron, Q., Grenfell, B.T., Levin, S.A. and Laxminarayan, R., (2014). Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data. *The Lancet Infectious Diseases*, 14(8), pp.742-750.

Vijayakumar, S. and Ganesan, S., (2012). In vitro cytotoxicity assay on gold nanoparticles with different stabilizing agents. *Journal of Nanomaterials*, 14.

Walsh, C., (2000). Molecular mechanisms that confer antibacterial drug resistance. *Nature,* 406, 775-781.

Wang, H., Chen, Y., Li, X.Y. and Liu, Y., (2007). Synthesis of oligo (ethylenediamino)-*β*-cyclodextrin modified gold nanoparticle as a DNA concentrator. *Molecular pharmaceutics*, 4(2), pp.189-198.

Wangchuk, P., (2014). Phytochemical analysis, bioassays and the identification of drug lead compounds from seven Bhutanese medicinal plants.

Wannes, W.A., Mhamdi, B., Sriti, J., Jemia, M.B., Ouchikh, O., Hamdaoui, G., Kchouk, M.E. and Marzouk, B., (2010). Antioxidant activities of the essential oils and methanol extracts from myrtle (*Myrtus communis* var. *italica L.)* leaf, stem and flower. *Food and chemical toxicology*, 48(5), pp.1362-1370.

Weir, E., Lawlor, A., Whelan, A. and Regan, F., (2008). The use of nanoparticles in anti-microbial materials and their characterization. *Analyst,* 133(7), pp.835-845.

Wood, A.J., Gold, H.S. and Moellering, J.R, (1996). Antimicrobial-drug resistance. New England *Journal of Medicine*, 335, 1445-1453.

World Health Organization. (2016). Antimicrobial resistance. Global Antimicrobial Resistance journal surveillance. Geneva: WHO Press.

Yan, J.J., Zheng, P.X., Wang, M.C., Tsai, S.H., Wang, L.R. and Wu, J.J., (2015). Allocation of *Klebsiella pneumoniae* blood stream isolates into four distinct groups by ompK36 typing in a Taiwanese university hospital. *Journal of clinical microbiology*, 53(10), pp.3256-3263.

Zetola, N., Francis, J.S., Nuermberger, E.L. and Bishai, W.R., (2005). Community-acquired meticillin-resistant *Staphylococcus aureus*: an emerging threat. *The Lancet infectious diseases*, 5(5), pp.275-286.

Zhao, Y., and Jiang, X., (2013). Multiple strategies to activate gold nanoparticles as antibiotics. *Nanoscale* 5, 8340–8350.doi:10.1039

CHAPTER THREE: EVALUATION OF ANTIMICROBIAL ACTIVITIES OF EXTRACT FROM *PYRENACANTHA GRANDIFLORA* BAILL (ICACINACEAE)

3.1 SUMMARY

BACKGROUND: Microbial drug resistance is a growing health problem. This has led to search for new antimicrobial compound and plants are considered as one of the most promising sources for new antimicrobials discovery. *Pyrenacantha grandiflora* (*P. grandiflora*) Baill is used for the treatment and management of diarrhea, gastrointestinal-related infections, dysentery, inflammation and tooth pain by traditional healers in the Venda region.

OBJECTIVE: The objective of the present study was to evaluate the antimicrobial activity of *P. grandiflora t*ubers using different extraction solvents against 15 bacterial and 11 fungal strains.

MATERIALS AND METHODS: Plant extracts were obtained using 5 solvents separately, boiled water, cold water, methanol, dichloromethane, chloroform and ethyl acetate. Hole plate assay was used for initial evaluation of antimicrobial properties of plant materials. Minimum inhibitory concentrations (MIC) of the most active plant extracts were determined by the broth microdilution method. One-way ANOVA was used for data analysis.

RESULTS: The hole plate assay revealed that the highest antibacterial activity was against *Micrococcus kristinae* with ethyl acetate extract and no extract was active against *Candida* and *Fusarium* species using this method. The MIC of the extracts was determined and all the extracts showed antimicrobial activity against all tested strains ranging from 0.06-7.5 mg/ml. Some extracts appeared to be fungicidal and hot water extracts were more active against *Cryptococcus neoformans* with the MFC value of 0.06 mg/ml Methanol extract was also active against most test

strains including *Candida tropicalis* with the minimum fungicidal concentration value of 3.75 mg/ml.

CONCLUSION: *Pyrenacantha grandiflora* contains bioactive substances that make it active against bacterial and fungal pathogens. This is the first time the antimicrobial and antifungal activities of *P. grandiflora* have been demonstrated scientifically. Extraction with hot water as done by the traditional healers showed activity thereby justifying the traditional use of this plant.

Keywords: Antibacterial, antifungal, *Pyrenacantha,* MIC, MFC, bacterial and fungal species

3.2 INTRODUCTION

World Health Organization (WHO) 2014 report on global surveillance of antimicrobial resistance revealed that antibiotic resistance is no longer a prediction for the future, it is happening right now, across the world and the ability to treat common infections in the community and hospitals is at risk. Therefore, the outlook for the use of antimicrobial drugs in the future is still uncertain (Nascimento *et al*., 2000). There is an urgent need for new antimicrobials or any action to effectively fight against resistant microbes. Furthermore, the search of new antimicrobial compounds should be done to better comprehend their properties, safety and efficiency/ effectiveness (Ellof, 1998).

Plants are considered to be amongst the safest sources because of their natural origin as compared to the synthetic compounds (Rajeh *et al*., 2010; Upadhyay *et al*., 2014). Plant secondary metabolites are known to be responsible for antimicrobial action. Other advantages of using plant-derived antimicrobial include the fact that they have several target sites and have a variety of mechanisms of action (Upadhyay *et al*., 2014; Ahmad and Beg, 2001; Cowan, 1999; Petrosyan *et al*., 2015). Several studies have highlighted the antibacterial activities of South African traditional medicinal plants (Obi *et al*., 2003; Ranasamy *et al*., 2007; Samie *et al*., 2007; Gundidza *et al*., 2009). However, *Pyrenacantha grandiflora* has not been studied.

Pyrenacantha grandiflora Baill in a medicinal plant used by elders in the Venda region in Limpopo, South Africa. It is known by the common names Gwere, Velavhahleka, Mbengelele (in Tshivenda) and Velabahleke (Siswati). *Pyrenacantha grandiflora* belongs to the family Icacinaceae. There are approximately 48 species, subspecies, varieties, forms and cultivars in the genus *Pyrenacantha*. Members of the genus mostly studied includes *Pyrenacantha staudtii* which was found to possess an inhibitory activity of the derivatives of 3-Carbomethoxypyridine on isolated rat uterus (Nworgu *et al.*, 2007).

Although not many specific indications have been seen for this species, it belongs to a genus where most, if not all members of the genus produce hydrogen cyanide, a toxic substance that gives almonds their characteristic flavor and it is typically present in too little amount to do any damage (Tshibangu *et al*., 2002). In small quantities, produced hydrogen cyanide has been appeared to stimulate respiration and improve digestion, it is also asserted to be of advantage in the treatment of cancer. In abundance, in any case, it can cause respiratory failure and even death (Krochmal and Krochmal, 1984).

In the Venda region of South Africa, *Pyrenacantha grandiflora* is used for the treatment and management of gastroenteritis, dysentery, inflammation and tooth pain (Obi *et al*., 2007). Apart from the antimicrobial activities, plant extracts have also exhibited immunomodulatory effects on various cell cultures and in experimental animals (Nakamura *et al*., 1999; Tona *et al*., 2003). Previously, Ramalivhana and colleagues (Obi *et al*., 2007). Conducted a study to determine the antimicrobial activities of *Pyrenacantha grandiflora* Baill and *Ficus sycomorus* used by traditional healers in Limpopo province against *Aeromonas* spp. However, there is a dearth of information on the antimicrobial activity of extract from the genus *Pyrenacantha*. The objective of the current research was to evaluate the antimicrobial activities of extracts from *Pyrenacantha grandiflora* prepared using different solvents.

3.3 MATERIALS AND METHODS

3.3.1 Microorganism and growth conditions

Following microorganisms were used as test strains, *Streptococcus faecalis* (ATCC 29212), *Staphylococcus epidermidis* (clinical isolate), *Staphylococcus aureus* (clinical isolate), *Pseudomonas aeruginosa* (ATCC 19582), *Serratia marsecens* (ATCC 9986), *Klebsiella*

pneumonia (clinical isolate), *Acinetobacter calcoaceticus* , *Escherichia coli* (clinical isolate), *Micrococcus kristinae* (clinical isolate), *Escherichia coli* (ATTC 8739), *Proteus vulgaris* (ATCC 6830), *Pseudomonas aeruginosa* (ATCC 7700), *Salmonella spp.* (clinical isolate), *Salmonella typhi* (clinical isolate), *Candida krusei*, *Candida tropicalis*, *Candida albicans*, *Candida parapsilosis*, *Candida glabrata*, *Cryptococcus neoformans*, *Fusarium oxysporum*, *Fusarium graminearum, Fusarium nygamai*, *Fusarium verticillioides* , *Fusarium proliferatum*. Mueller-Hinton broth medium (MHB) (Rochelle chemical, SA) and Mueller-Hinton agar medium (MHA) (Rochelle chemicals, SA) were used to cultivate bacteria and Mueller-Hinton broth medium and Sabouraud dextrose agar were used for yeast cultivation.

3.3.2 Collection, identification and drying of plant materials

Plants material collection was done in October 2014 a village called Masisi situated at about 30 km outside of Thohoyandou. The tubers were harvested and were washed with tap water to remove any contaminants and subsequently placed in a drying room for 2 weeks. Dried plant materials were finely grounded into powder and stored in a sealed glass jar at room temperature.

3.3.3 Preparation of plant crude extract

Plant materials were extracted using different solvents which included boiled and cold water, chloroform, methanol, ethyl acetate and dichloromethane extract (Rochelle chemical, SA). For boiled water extract, the stock solution was prepared by adding 50 g of crude dried tuber in 500 ml of distilled water then boiled for 15 min followed by cooling and kept at 4^{o}C. For cold water extraction, chloroform, methanol, ethyl acetate and dichloromethane extract, 50 g of dried tuber was added to 500 ml of the respective solvent and allowed to homogenize for 3 days at room

temperature. The mixture was filtered through 22 µm filter and the extracts were further dried using a rotary evaporator.

3.3.4 Hole plate assay

Antimicrobial activity of *P. grandiflora* was initially evaluated using hole plate assay (Balouiri *et al.*, 2016). Growth medium was poured into Petri dishes at 50-70^{o}C and left to solidify under ultraviolet (UV) light (265 nm wavelength) for about an hour. A sterile cotton swab was dipped into an overnight culture of each bacterial strain (adjusted to the turbidity of 0.5 McFarland standard). An agar plate was inoculated by evenly streaking cotton swab over the agar medium surface. Small holes with a diameter of 8 mm were punched in the medium with a sterile cock borer and 30 µl of the extracts (of 100 mg/ml) were dispensed into the holes and the plates were incubated at 37^{o}C for 24 h. The diameter of growth inhibition zone around the holes were measured.

3.3.5 Microdilution assay

The microdilution method was used to determine the minimum inhibitory concentrations (MICs) of the plant extracts using 96 well microtitration plates (Samie *et al*., 2005). Briefly, 185 µl of the broth was added to each well in the first row of the microtitration plate and 100 µL to the rest of the wells from the second row downwards. Plant crude extracts (15 µl) was then added into each well on the first row (row A), starting with the positive control penicillin (Sigma, Germany), followed by the negative control (20% DMSO (Sigma-Aldrich, Germany) used to dissolve the plant extracts) and the plant extracts in the rest of the wells in that row. A two-fold serial dilution was done by mixing the contents in each well of the first row and transferring 100 µl to the next well of

the same column and the same procedure was done up to the last well of the same column and the last 100 µl from the last well was discarded. Then 100 µl of different microorganism suspensions was added and the plate was incubated overnight at 37ºC. The next day, 40 µl of INT (Iodo-nitro tetrazolium) was added in each well and the plate was incubated for 10 min. After 10 min incubation, the results were read using a spectrophotometer (SpectraMax M2, Sunnyvale, USA), observing the color change and determining the MIC. All the extract that showed activity (no color change) were inoculated again on the agar plate and incubated overnight to determine the MBC and MFC.

3.3.6 Determination of MBC/MFC values

Minimum bactericidal concentration (MBC) and minimum fungicidal concentrations (MFC) of the plant crude extracts were determined by sub-culturing the samples (5 µl) taken from the wells without growth during MIC determination on the agar medium. The lowest concentration of crude extracts with the absence of growth after 24 h incubation at 37ºC was considered as MBC/MFC.

3.3.7 Growth inhibition assay

Growth inhibition assay for 3 extracts including methanol, cold and hot water was conducted to evaluate the antimicrobial activity of *P. grandiflora* against *Fusarium* species. Different plant extracts were inoculated in the growth medium of *Fusarium* species and the diameters of inhibition of fungal growth were recorded daily for 7 days.

3.3.9 Statistical analysis

All the data were loaded onto access and one way ANOVA was used for statistical analysis. The difference was considered statistically significant if the $p<0.05$.

3.4 RESULTS

The activity of the extracts from *P. grandiflora* prepared with solvents such as boiling and cold water, chloroform, methanol, ethyl acetate and dichloromethane were evaluated against different microorganism including bacteria and fungi (*Candida spp., Cryptococcus neoformans* and *Fusarium spp*.).

3.4.1 Hole plate assay

The antimicrobial assay of different plant extracts was initially evaluated using hole plate assay. The hole plate assay revealed that the ethyl acetate extract had the highest antibacterial activity showing a diameter of inhibition of 18 mm against *Micrococcus kristinae.* Dichloromethane and chloroform extracts showed no activity against all bacteria. Methanol extract appeared to inhibit the growth of most bacteria with the highest diameter of 15 mm against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (**Table 3.1**). There was no extract active against *Candida* and *Fusarium* species using the hole plate assay.

Table 3.1: Antibacterial activity of *P. grandiflora* using the hole plate method.

Organisms	**Methanol extract**	**Hot water extract**	**Cold water extract**	**Dichloromethane extract**	**Chloroform extract**	**Ethyl acetate extract**
Streptococcus faecalis (ATCC 29212	8	12	0	0	0	0
Staphylococcus epidermidis (clinical isolate)	12	10	10	0	0	0
Staphylococcus aureus (Clinical isolate)	15	0	0	0	0	0
Pseudomonas aeruginosa (ATCC 19582)	15	0	15	0	0	10
Serratiamarsecens (ATCC 9986)	8	0	8	0	0	10
Klebsiella pneumonia (clinical isolate)	12	0	0	0	0	10
Acinetobacter calcoaceticus	13	0	0	0	0	5
Escherichia coli (clinical isolate)	10	15	15	0	0	0
Micrococcus kristinae (Clinical isolate)	0	10	0	0	0	18
Escherichia coli (ATCC 8739)	0	0	0	0	0	7
Proteus vulgaris (ATCC6830)	7	10	8	0	0	0
Shigellaflexneri (Clinical isolate)	0	5	0	0	0	0
Pseudomonas aeruginosa (ATCC 7700).	0	8	0	0	0	14
Salmonella spp (clinical isolate)	0	10	0	0	0	0
Salmonella typhi (Clinical isolate)	0	0	5	0	0	0

The amount of extracts was 30µl. The results are presented as diameters of inhibition zones in mm.

3.4.2 Microdilution assay

The minimum inhibition concentration was determined by broth microdilution method. The most active plant extract using this method was chloroform extract with the concentration of 0.12 mg/ml against *Escherichia coli*. Ethyl acetate extracts had the lowest overall MIC value compared to all the other extracts. Dichloromethane showed the highest MIC value against all different bacteria (**Table 3.2**).

Table 3.2: Minimum inhibitory concentration (MIC) of different bacteria with microdilution method.

Organisms	**Methanol extract**	**Hot water extract**	**Cold water extract**	**Dichloromethane extract**	**Chloroform extract**	**Ethyl acetate extract**
Streptococcus faecalis (ATCC 29212	0.95mg/ml	0.95mg/ml	0.95mg/ml	>7.5mg/ml	>7.5mg/ml	1.9 mg/ml
Staphylococcus epidermidis (clinical isolate)	>7.5mg/ml	7.5mg/ml	3.75mg/ml	>7.5mg/ml	>7.5mg/ml	3.75mg/ml
Staphylococcus aureus (Clinical isolate)	0.06mg/ml	1.9 mg/ml	0.95mg/ml	>7.5mg/ml	>7.5mg/ml	3.75mg/ml
Pseudomonas aeruginosa (ATCC 19582)	3.75mg/ml	7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	3.75mg/ml
Serratiamarsecens (ATCC 9986)	1.9 mg/ml	7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml
Klebsiella pneumonia (clinical isolate)	0.48mg/ml	1.9 mg/ml	3.75mg/ml	>7.5mg/ml	>7.5mg/ml	3.75mg/ml
Acinetobacter calcoaceticus(Clinical isolate)	>7.5mg/ml	>7.5mg/ml	7.5mg/ml	>7.5mg/ml	>7.5mg/ml	7.5mg/ml
Escherichia coli (clinical isolate)	>7.5mg/ml	7.5mg/ml	7.5mg/ml	>7.5mg/ml	0.12mg/ml	3.75mg/ml
Micrococcus kristinae (Clinical isolate)	1.9 mg/ml	0.12mgml	7.5mg/ml	>7.5mg/ml	>7.5mg/ml	3.75mg/ml
Escherichia coli (ATCC 8739)	>7.5mg/ml	7.5mg/ml	7.5mg/ml	>7.5mg/ml	>7.5mg/ml	3.75mg/ml
Proteus vulgaris (ATCC 6830)	>7.5mg/ml	7.5mg/ml	7.5mg/ml	>7.5mg/ml	>7.5mg/ml	3.75mg/ml
Shigellaflexneri(Clinical isolate)	>7.5mg/ml	7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	3.75mg/ml
Pseudomonas aeruginosa (ATCC 7700)	>7.5mg/ml	7.5mg/ml	3.75mg/ml	>7.5mg/ml	7.5mg/ml	3.75mg/ml
Salmonella spp (clinical isolate)	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	3.75mg/ml
Salmonella typhi (Clinical isolate)	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	7.5mg/ml

The methanol extract was the most active with the MIC value of 0.12 mg/ml against *Candida parapsilosis*. Ethyl acetate extract showed the lowest MIC overall value against all *Candida* spp. and *Cryptococcus neoformans*. Dichloromethane and chloroform extract were least active with MIC values 7.5 mg/ml against all *Candida* spp. and *Cryptococcus neoformans* (**Table 3.3**).

Table 3.3: Minimum inhibitory concentration (MIC) of *P. grandiflora* against yeast using microdilution method.

Organisms	**Methanol extract**	**Hot water extract**	**Cold water extract**	**Dichloromethane extract**	**Chloroform extract**	**Ethyl acetate extract**
Candidakrusei	3.75mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	3.75mg/ml
Candida tropicalis	3.75mg/ml	>7.5mg/ml	7.5mg/ml	>7.5mg/ml	>7.5mg/ml	3.75mg/ml
Candida albicans	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	3.75mg/ml
Candida parapsilosis	0.12mg.ml	0.95mg/ml	7.5mg/ml	>7.5mg/ml	>7.5mg/ml	3.75mg/ml
Candida glabrata	3.75mg/ml	3.75mg/ml	7.5mg/ml	>7.5mg/ml	>7.5mg/ml	3.75mg/ml
Cryptococcus neoformans	>7.5mg/ml	0.06mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	3.75mg/ml

Fusarium species were also used in the evaluation of *P. grandiflora* activity using broth microdilution method. The methanol extract was the most active with the MIC value of 0.06 mg/ml against *Fusarium nygamai*. Cold water extract showed the lowest MIC overall value in all *Fusarium spp*. Dichloromethane and chloroform extract were less active with MIC value of 7.5 mg/ml against all *Fusarium spp*. (**Table 3.4**).

Table 3.4: Minimum inhibitory concentration of *P. grandiflora* against *Fusarium* species using microdilution method.

Organisms	Methanol extract	Hot water extract	Cold water extract	Dichloromethane extract	Chloroform extract	Ethyl extract
Fusarium oxysporum	1.9mg/ml	7.5mg/ml	1.9mg/ml	>7.5mg/ml	>7.5mg/ml	3.75mg/ml
Fusarium graminearum	7.5mg/ml	7.5mg/ml	7.5mg/ml	>7.5mg/ml	>7.5mg/ml	3.75mg/ml
Fusarium nygamai	0.06mg/ml	3.75mg/ml	0.95mg/ml	>7.5mg/ml	>7.5mg/ml	3.75mg/ml
Fusarium verticilliaides	1.9mg/ml	7.5mg/ml	1.9mg/ml	>7.5mg/ml	>7.5mg/ml	3.75mg/ml
Fusarium proliferatum	7.5mg/ml	7.5mg/ml	3.75mg/ml	>7.5mg/ml	>7.5mg/ml	3.75mg/ml

3.4.3 Determination of MBC/MFC values

All the wells containing extracts that showed activity (no color change) during microdilution assay were inoculated again in the agar to check if they can kill the microorganisms. Most extracts were active against the organisms tested and hot water extract appeared to be more active against *C. neoformans* with the MFC value of 0.06 mg/ml. Methanol extract also showed to be active against *C. tropicalis* with the MFC value of 3.75 mg/ml (**Table 3.5**). Cold water, dichloromethane, chloroform and ethyl acetate extracts were not fungicidal to any of the fungal organism tested. None of the extracts was bactericidal and none of the extracts was fungicidal against the fusarium species.

Table 3.5: Minimum fungicidal concentration (MFC) using microdilution method.

Organisms	Methanol extract	Hot water extract	Cold water extract	Dichloromethane extract	Chloroform extract	Ethyl acetate extract
Candida krusei	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml
Candida tropicalis	**3.75mg/ml**	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml
Candida albicans	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml
Candida parapsolosis	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml
Candida glabrata	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml
Cryptococcus neoformans	>7.5mg/ml	**0.06mg/ml**	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml	>7.5mg/ml

3.4.4 Growth inhibition assay

Growth inhibition assay of three extracts including methanol, cold and hot water was conducted to evaluate the cytotoxicity of *P. grandiflora* against *Fusarium* species. The methanol extract was observed to be most active against *Fusarium verticillioides* (**Fig. 3.1a**), *Fusarium oxysporum* (**Fig. 1b**) and *Fusarium proliferatum* (**Fig. 1c**) with the lowest diameter (<25 mm) of fungal growth compared to other extract during seven days of evaluation (**Fig. 3.1**). Cold water and methanol extracts were both active against *Fusarium graminearum* (**Fig. 1d**) with the diameter of 25 mm of fungal growth on the last 3 days. All extracts were active against *Fusarium nygamai* (**Fig. 1e**).

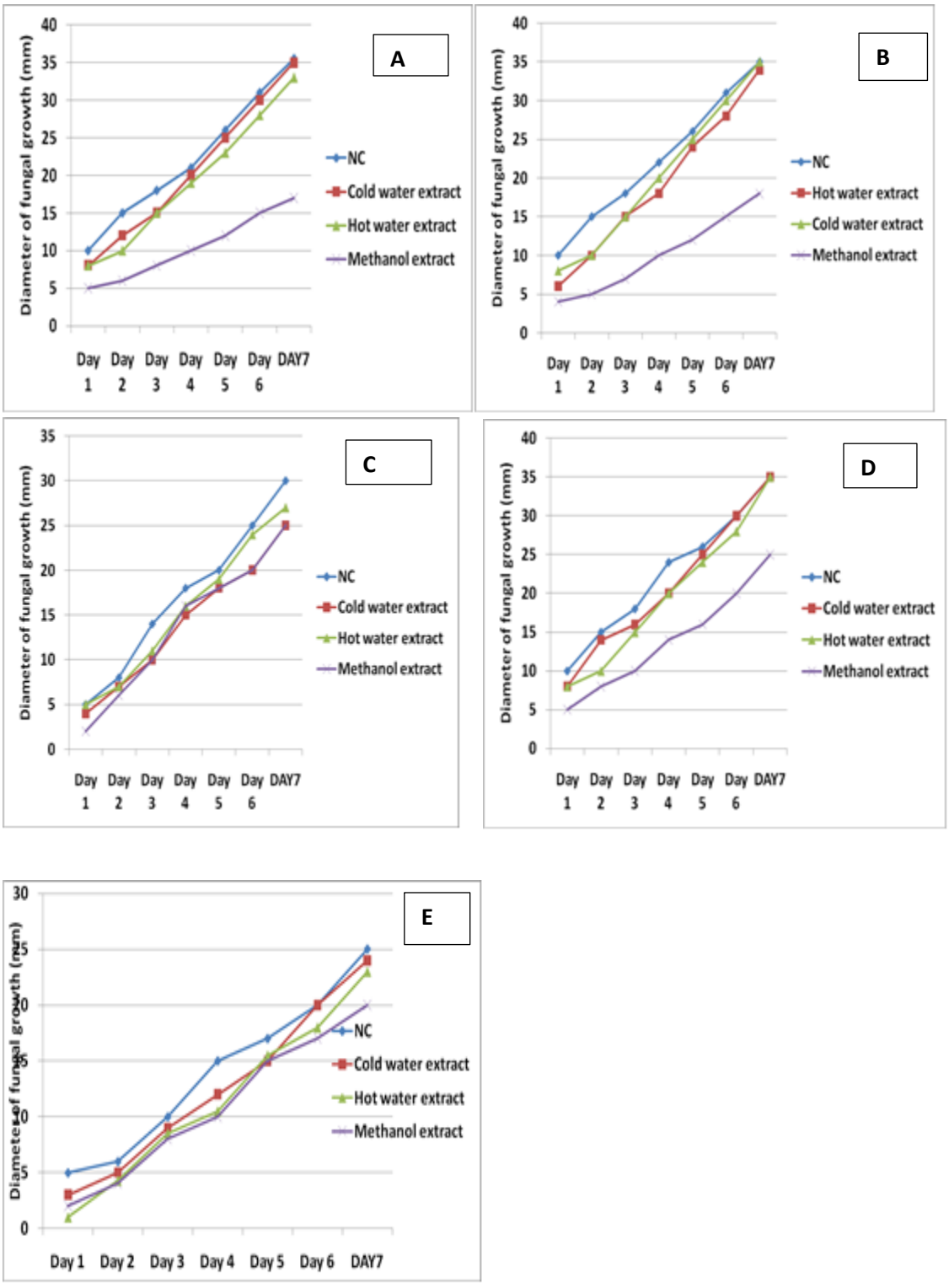


Figure 3.1. Growth inhibition assay of 3 extracts against *Fusarium verticilloides* (A); *Fusarium oxysporum* (B); *Fusarium proliferatum*(C); *Fusarium graminearum* (D); and *Fusarium nygamai*' (E) ($p<0.05$).

3.5 DISCUSSION

It is found that *P. grandiflora* extract has antimicrobial activity against numerous bacteria and fungi as this was confirmed using hole plate assay as well as microdilution assay. Furthermore, *P. grandiflora* extract was able to inhibit the growth of bacteria and fungi. However, they were not able to kill the bacteria whereas fungicidal activity was observed. Since different solvents were used during extraction of plant material, methanol extract was the most effective showing threpeautic properties amongst them all. The present study is the first attempt to determine the antimicrobial activities *of P. grandiflora* against organisms other than *Aeromonas spp* (Obi *et al*., 2007).

The study showed that water extracts as well as methanol extracts were the most active against some of the microorganisms tested particularly when the microdilution method was used. Their activities against bacteria and fungi that affect humans and plants illustrated that this plant could be very useful in the identification of compounds that could serve as lead in the development of drugs for the treatment of human, animal and plant diseases.

The study of other species of the genus *Pyrenacantha* showed that the leaves of *Pyrenacantha staudtii* are used as antimalarial remedies in the Democratic Republic of Congo (Mesia *et al*., 2005). After plant collection, the tubers were dried since Mendes et al (2013) suggested that drying is very important because it causes loss of volatile antimicrobials (peroxide, terpenoid and bromo-ether compounds and volatile fatty acid) present in the fresh plant (Mendes *et al*., 2013). Subsequently, washing the tuber to remove soil and other contaminants then ground into powder for easier further analysis is done.

Different solvents were used during extraction in this study, this includes boiled water since drying followed by boiling increased the phytochemicals content and enhanced the inhibitory potential (Águila-Ramírez *et al*., 2012). Probably, inhibition mechanisms are due in part to the hydrophobic

nature of some components, such as fatty products (Plaza *et al*., 2010; Adaikalaraj *et al*., 2012). Other studies confirmed that polar extracts have higher antibacterial activity (Jassbi *et al*., 2013; De Jesus Raposo *et al.*, 2015; Hellio *et al*., 2001). Systematical evaluation and optimization of the solvent is necessary for accurate and reproducible preparation of extracts. Several studies aimed at selecting the best solvent, which was usually one of the following, water, methanol, ethanol, acetone, ethyl acetate, dichloromethane, chloroform, diethyl ether and hexane (Suleiman *et al*., 2010; Krish and Das, 2014).

The antimicrobial assay of different extracts was initially evaluated using hole plate assay and all extracts were active against bacteria only. This is an indication that the compounds active against these bacterial organisms are probably polar. Other authors have found that the polarity of the compounds was important in determining the biological activity and many non-polar compounds were active against a number of bacterial organisms (Ahmed *et al*., 2014). Fungi (*Candida* and *Fusarium* species) are characterized by having the cell wall which might not be easily accessed by polar compounds. This could explain why none of the extract were effective against the fungi when the hole plate assay was used. Methanol extracts generally inhibit most microorganism (Águila-Ramírez *et al.*, 2012; Plaza *et al*., 2010).

In this study hole plate assay was performed and methanol extracts inhibited the growth of most bacteria with the diameter of 15 mm against *S. aureus* and *P. aeruginosa* whereas highest antibacterial activity diameter of 18 mm zone of growth inhibition was observed with ethyl acetate extract against *Micrococcus kristinae*. In another study, it was found that methanol extracts had good activities against *P. aeruginosa* when the microdilution method was used (Voukeng *et al*., 2017). Dichloromethane and chloroform extract showed no activity against all bacteria this may due to their low level of polarity as compared to other solvents used in this study or any other physical or chemical characteristic.

The MIC values obtained from our results ranged from 0.06-7.5 mg/ml. The MIC values were lower than those obtained with organic extracts of the *Kirkia wilmsii* leaves which ranged from 0.17-2.11 mg/ml (Rios *et al*., 1998). However, 7.5 mg/ml was much higher than the reported maximum of 2.11 mg/ml in the previous study. This comparison shows that the tuber of *P. grandiflora* might be more potent. Our findings showed that the tuber extracts exhibited strong antimicrobial activity with the smallest MIC of 0.06 mg/ml which was smaller than the concentration of 1 mg/ml previously reported (Vatsos and Rebours, 2015), a concentration at which the plant extracts are considered to have good potency of antimicrobial level when using the micro-plate dilution method (Balouiri *et al*., 2016). Therefore, these extracts should be pursued as they could be a good source of the bioactive compound. *Pyrenacantha grandiflora* did not show any bactericidal activity. However, Ramalivhana and colleagues (Obi *et al.*, 2007) and others obtained low bactericidal activity with *P. grandiflora* compared to *F. sycomorus*. For future study, other solvent such as ethanol and diethyl ether may also be used during extraction.

Growth inhibition assay of three extracts was conducted against *Fusarium species*. In most cases, the extracts prepared using organic solvents appeared more efficient and this is similar to previous studies (Saritha *et al*., 2013; Rajauria *et al*., 2013). Abundant studies have confirmed that alcoholic solutions and/or hydrophilic solvent mixtures provided better activity, i.e., methanol and acetone extracts were more active than those in lipophilic solvents such as chloroform/methanol (Mendes *et al*., 2013; Rosaline 2012). In this study, methanol extract was observed to be most active against *Fusarium verticillioides* while *Fusarium oxysporum* and *Fusarium proliferatum* showed the lowest diameter of fungal growth which was significant ($p<0.05$) compared to other extracts on day seven of evaluation. Rajauria et al., 2013 found considerable variations in the extraction yield and antimicrobial activity among different concentrations of methanol extract. Cold water and methanol extracts were both active against *Fusarium graminearum* with the diameter of 25 mm of fungal growth on the last days. However,

the optimal solvent depends on many factors, particularly on the target solutes and microorganisms (Ginovyan *et al*., 2017). All extracts were active against *Fusarium nygamai* (E).

3.6 CONCLUSION

The present study validated the efficacy of *P. grandiflora* which is used in traditional medicine. The results revealed that water extracts which is generally used by the traditional healers is active against most microorganisms tested as well as methanol extract. Further study should be conducted to purify the plants' active compounds responsible for their antimicrobial action and examine their efficiency on other bacteria, fungi and also helminths. Synergistic effect of these plant extracts with commonly used antibiotics will also be interesting to explore. However, apart from antimicrobial activities, these plant extracts could also be exploited to cure several disorders. The results of present investigation clearly indicate that antimicrobial activity of *Pyrenacantha grandiflora* Baill tuber varies with test strain and the type of solvent used during extraction, this clearly gives hope for future development of drug leads.

3.7 *REFERENCES*

Adaikalaraj, G., Patric, R.D., Johnson, M., Janakiraman, N. and Babu, A., (2012). Antibacterial potential of selected red seaweeds from Manapad coastal areas, Thoothukudi, Tamil Nadu, India. *Asian Pacific Journal of Tropical Biomedicine*, 2(2), pp.S1077-S1080.

Águila-Ramírez, R.N., Arenas-González, A., Hernández-Guerrero, C.J., González-Acosta, B., Borges-Souza, J.M., Véron, B., Pope, J. and Hellio, C., (2017). Antimicrobial and antifouling activities achieved by extracts of seaweeds from Gulf of California, Mexico. *Hidrobiológica*, 22(1), pp.8-15.

Ahmad, I. and Beg, A.Z., (2001). Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. *Journal of ethnopharmacology*, 74(2), pp.113-123.

Ahmed, A.S., McGaw, L.J., Elgorashi, E.E., Naidoo, V. and Eloff, J.N., (2014). Polarity of extracts and fractions of four Combretum (Combretaceae) species used to treat infections and gastrointestinal disorders in southern African traditional medicine has a major effect on different relevant in vitro activities. *Journal of ethnopharmacology*, 154(2), pp.339-350.

Balouiri, M., Sadiki, M. and Ibnsouda, S.K., (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, 6(2), pp.71-79.

Cowan, M.M., (1999). Plant products as antimicrobial agents. *Clinical microbiology reviews*, 12(4), pp.564-582.

de Jesus Raposo, M.F., de Morais, A.M.B. and de Morais, R.M.S.C., (2015). Marine polysaccharides from algae with potential biomedical applications. *Marine drugs*, 13(5), pp.2967-3028.

Eloff, J.N., (1998). Which extractant should be used for the screening and isolation of antimicrobial components from plants. *Journal of ethnopharmacology*, 60(1), pp.1-8.

Ginovyan, M., Petrosyan, M. and Trchounian, A., (2017). Antimicrobial activity of some plant materials used in Armenian traditional medicine. *BMC complementary and alternative medicine*, 17(1), pp.50.

Gundidza, M., Gweru, N., Mmbengwa, V., Ramalivhana, N.J., Magwa, Z. and Samie, A., (2008). Phytoconstituents and biological activities of essential Oil from *Rhus lancea* L. F. *African Journal of Biotechnology*, 7(16).

Hellio, C., D. De La Broise, L. Dufosse, Y. Le Gal and N. Bourgougnon, (2001). Inhibition of marine bacteria by extracts of macroalgae: Potential use for environmentally friendly antifouling paints. *Marine Environmental Research*., 52: 231-247.

Jassbi, A.R., Mohabati, M., Eslami, S., Sohrabipour, J. and Miri, R., (2013). Biological activity and chemical constituents of red and brown algae from the Persian Gulf. *Iranian journal of pharmaceutical research*: IJPR, 12(3), p.339.

Krish, S. and A. Das, (2014). In-vitro bioactivity of marine seaweed, Cladophora rupestris. *International Journal of Pharmaceutics Biological Sciences*. 5: pp. 898-908.

Krochmal, A. and Krochmal, C., (1984). A field guide to medicinal plants. New York: Times Books vii, 274p.-illus. En Icones. Geog Systematics: Angiospermae (KR, 198502499).

Mendes, M., R. Pereira, I.S. Pinto, A.P. Carvalho and A.M. Gomes, (2013). Antimicrobial activity and lipid profile of seaweed extracts from the North Portuguese coast. *International Food Research Journal*, 20: 3337-3345.

Mesia, G.K., Tona, G.L., Penge, O., Lusakibanza, M., Nanga, T.M., Cimanga, R.K., Apers, S., Van Miert, S., Totte, J., Pieters, L. and Vlietinck, A.J., (2005). Antimalarial activities and toxicities

of three plants used as traditional remedies for malaria in the Democratic Republic of Congo: Croton mubango, *Nauclea pobeguinii* and *Pyrenacantha staudtii*. *Annals of Tropical Medicine & Parasitology*, 99(4), pp.345-357.

Nakamura, C.V., Ueda-Nakamura, T., Bando, E., Melo, A.F.N., Cortez, D.A.G. and Dias Filho, B.P., (1999). Antibacterial activity of *Ocimum gratissimum L*. essential oil. *Memórias do Instituto Oswaldo Cruz*, 94(5), pp.675-678.

Nascimento, G.G., Locatelli, J., Freitas, P.C. and Silva, G.L., (2000). Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. *Brazilian journal of microbiology*, 31(4), pp.247-256.

Nworgu, Z.A., Falodun, A. and Usifoh, C.O., (2007). Inhibitory Effect Of 3-Carbomethoxypyridine and 3-Carbobutoxypyridine On Isolated Rat Uterus*. Acta poloniae pharmaceuticatical*, 64(2), pp.179-182.

Obi, C.L., Potgieter, N., Bessong, P.O., Masebe, T., Mathebula, H., Molobela, P. and Jäger, A.K., (2003). *In vitro* antibacterial activity of Venda medicinal plants. *South African journal of botany*, 69(2), pp.199-203.

Obi, C.L., Ramalivhana, J., Samie, A. and Igumbor, E.O., (2007). Prevalence, pathogenesis, antibiotic susceptibility profiles, and in-vitro activity of selected medicinal plants against *Aeromonas* isolates from stool samples of patients in the Venda region of South Africa. *Journal of health, population, and nutrition,* 25(4), pp.428.

Petrosyan, M., Shcherbakova, Y., Sahakyan, N., Vardanyan, Z., Poladyan, A., Popov, Y. and Trchounian, A., (2015). Alkanna orientalis (L.) Boiss. plant isolated cultures and antimicrobial activity of their extracts: phenomenon, dependence on different factors and effects on some membrane-associated properties of bacteria. Plant Cell, Tissue and Organ Culture (PCTOC), 122(3), pp.727-738.

Plaza, M., Santoyo, S., Jaime, L., Reina, G.G.B., Herrero, M., Señoráns, F.J. and Ibáñez, E., (2010). Screening for bioactive compounds from algae. *Journal of pharmaceutical and biomedical analysis*, 51(2), pp.450-455.

Rajauria, G., Jaiswal, A.K., Abu-Gannam, Nissre, E.N., and Gupta, S., (2013). Antimicrobial, antioxidant and free radical-scavenging capacity of brown seaweed *Himanthalia elongata* from western coast of Ireland. *Journal of Food Biochemistry*, 37(3), pp.322-335.

Rajeh, M.A.B., Z. Zuraini, S. Sasidharan, L.Y. Latha and S. Amutha, (2010). Assessment of *Euphorbia hirta* L. leaf, flower, stem and root extracts for their antibacterial and antifungal activity and brine shrimp lethality. *Molecules*, 15: 6008-6018.

Rangasamy, O., Raoelison, G., Rakotoniriana, F.E., Cheuk, K., Urverg-Ratsimamanga, S., Quetin-Leclercq, J., Gurib-Fakim, A. and Subratty, A.H., (2007). Screening for anti-infective properties of several medicinal plants of the Mauritians flora. *Journal of ethnopharmacology*, 109(2), pp.331-337.

Rios, J.L., Recio, M.C. and Villar, A., (1988). Screening methods for natural products with antimicrobial activity: a review of the literature. *Journal of ethnopharmacology*, 23(2-3), pp.127-149.

Rosaline, X.D., Sakthivelkumar, S., Rajendran, K. and Janarthanan, S., (2012). Screening of selected marine algae from the coastal Tamil Nadu, South India for antibacterial activity. *Asian Pacific Journal of Tropical Biomedicine*, 2(1), pp.S140-S146.

Samie, A., Obi, C.L., Bessong, P.O. and Namrita, L., (2005). Activity profiles of fourteen selected medicinal plants from Rural Venda communities in South Africa against fifteen clinical bacterial species. *African journal of Biotechnology*, 4(12).

Samie, A., Ramalivhana, J., Igumbor, E.O. and Obi, C.L., (2007). Prevalence, haemolytic and haemagglutination activities and antibiotic susceptibility profiles of *Campylobacter spp*. isolated from human diarrhoeal stools in Vhembe District, South Africa. Journal of health, population, and nutrition, 25(4), pp.406.

Saritha, K., Mani, A.E., Priyalaxmi, M. and Patterson, J., (2013). Antibacterial activity and biochemical constituents of seaweed *Ulva lactuca. Global Journal of Pharmacology*, 7(3), pp.276-282.

Savoia, D., (2012). Plant-derived antimicrobial compounds: alternatives to antibiotics. *Future microbiology*, 7(8), pp.979-990.

Suleiman, M.M., McGaw, L.J., Naidoo, V. and Eloff, J.N., (2010). Evaluation of several tree species for activity against the animal fungal pathogen *Aspergillus fumigatus*. *South African Journal of Botany*, 76(1), pp.64-71.

Tona, L., K. Kabul, N. Ngimbi and K. Cimanga, (2003). Evaluation of the antidiarrhoeal effects of Antiamoebic and phytochemical Spina-christi sterm bark in rats. *Acta Tropical journal*, 6: 1-5.

Tshibangu, J.N., Chifundera, K., Kaminsky, R., Wright, A.D. and König, G.M., (2002). Screening of African medicinal plants for antimicrobial and enzyme inhibitory activity. *Journal of Ethnopharmacology*, 80(1), pp.25-35.

Upadhyay, A., Upadhyaya, I., Kollanoor-Johny, A. and Venkitanarayanan, K., (2014). Combating pathogenic microorganisms using plant-derived antimicrobials: a minireview of the mechanistic basis. *BioMed research international*.

Vatsos, I.N. and Rebours, C., (2015). Seaweed extracts as antimicrobial agents in aquaculture. *Journal of applied phycology*, 27(5), pp.2017-2035.

Voukeng, I.K., Beng, V.P. and Kuete, V., (2017). Multidrug resistant bacteria are sensitive to Euphorbia prostrata and six others Cameroonian medicinal plants extracts. *BMC research notes*, 10(1), pp.321.

WHO, (2014). Antimicrobial Resistance: Global Report on Surveillance. World Health Organization, Geneva, Switzerland, and ISBN: 9789241564748, pp: 232.

CHAPTER FOUR: PHYTOCHEMICAL AND ANTIOXIDANT ANALYSIS OF *PYRENACANTHA GRANDIFLORA* (BAILL)

4.1 SUMMARY

BACKGROUND: Natural products, especially plant extracts are believed to be an important source of new chemical substances to provide unrestricted opportunities for new drug discoveries due to the development of adverse effects and microbial resistance to the chemically synthesized drugs.

OBJECTIVES: The aim of this study is to assess the phytochemical content, antioxidant and antimicrobial activities of *P. grandiflora* tuber extracts.

METHODS: Plant crude extraction was done using four solvents including methanol, chloroform, acetone and boiling water. Preliminary phytochemical screening was done and total phenolic content and total flavonoid content were quantified. Thin-layer chromatography and bioautography were used to isolate the phytochemicals and evaluate their antimicrobial activities. Infrared spectroscopy was used to determine the functional groups in the plant crude extracts, and antioxidant activity was determined using the DPPH scavenging assay.

RESULTS: *Pyrenacantha grandiflora* revealed the presence of phenolics, saponins, alkaloids, tannins, steroids, terpenoids and flavonoids in different proportions. The total phenolic content in the examined extracts ranged from 14.167 to 19.02 mg GA/g while the total flavonoid content in the examined extracts ranged from 26.603 to 34.621 mg QE/g. Thin-layer chromatography revealed various R_f values. The bioautography assay for antibacterial activity detection demonstrated well-defined inhibition zones against *E. coli* and *K. pneumonia* within the R_f value of 0.236. Spectroscopic studies of FTIR revealed different characteristic peak values with various functional compounds similar in all extracts but differ with transmittance values.

CONCLUSION: It was concluded that *P. grandiflora* extracts would directly lead to the establishment of some compounds that could be used to investigate new and more potent antimicrobials and antioxidants of the plant origin.

Keywords: Plant extracts, Phytochemicals, Phenolic content, Bioautography, Functional groups, Antioxidant activity

4.2 INTRODUCTION

Natural products especially those from medicinal plant extracts are regarded as an important source of novel chemical substances (Rakelly de Oliveira *et al*., 2015) that can provide unlimited opportunities for developing new medicines.The search and identification of medicinal plants for new compounds responsible for healing is needed due to the development of adverse effects and microbial resistance to the chemically synthesized drugs (Prabakara and Ravindran, 2011). The discoveries and development of novel compounds from medicinal plants has some advantages such as efficiency and cost effectiveness (Faidallah *et al*., 2016). According to the World Health Organization (WHO) (2016), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. During the last decade, infectious diseases and particularly infectious diarrhea have threatened the life of millions of people around the world especially patients with weak immune systems (Saha *et al*., 2018; Ashbolt, 2004). It is estimated that one in five children die before his/her fifth birthday due to diarrhea in developing countries (Haque *et al*., 2003).

In Africa, traditional medicine is still used as a primary health care source in curing several diseases, and in South Africa more than 70% of the population visit traditional healers concerning health issues (Kamanzy *et al*., 2002; Zuma *et al*., 2016). In Limpopo, traditional medicine is well recognized and different villages use numerous plants to treat gastrointestinal disorders such as diarrhea and infection by intestinal parasites, which are particularly prevalent in rural areas (McGaw *et al*., 2000). The Venda region of South Africa, situated in the northern part of the country, has a very strong tradition of medicinal plants (Samie *et al*., 2005) which are commonly used by the population. For example, *Pyrenacantha grandiflora* is used to cure gastrointestinal related disease, tooth pain and also diarrhea and its antibacterial and antifungal activities have been evaluated (Ramalivhana, 2010). However, no study has been done to characterize and identify its phytochemicals.

Phytochemicals are known as therapeutic chemical compounds that are found naturally in plants and responsible for healing purposes. About, 20,000 medicinal plants exist in 91 countries including 12 mega biodiversity countries. However, very few plants have been screened for phytochemicals (Sasidharan *et al*., 2011). Scientists estimate that there may be as many as 10,000 different phytochemicals having the potential to prove beneficial and have been reported to have biological activity such as anticancer, antimicrobial, antioxidant, antidiarrheal, and wound healing activity (Sasidharan *et al*., 2011). Identification of the chemical nature of phytochemical compounds present in the medicinal plants will provide some information on the different functional groups responsible for their medicinal properties.

4.3 MATERIALS AND METHODS

4.3.1 Chemicals and reagents

Methanol, acetone, ethanol, Dragendorff reagent and sodium hydroxide were purchased from Rochelle chemicals (RSA). Chloroform and hydrochloric acid was purchased at Merck (RSA). 2.2-Diphenyl-1-picrylhydrazyl (DPPH), and ascorbic acid were purchased from Sigma-Aldrich (St.Louis, USA). Aluminum chloride was purchased from Riedel-de Haen (Germany). Folin-Ciocalteu was purchase from Heyns laboratory (Fonteinbleau), Gallic acid and glacial acetic acid were purchased from Sigma- Aldrich (China).

4.3.2 Plant collection and extraction

Pyrenacantha grandiflora tubers were collected in cool dry condition. The harvested tubers were washed with distilled water to remove any contaminants. They were subsequently cut into smaller pieces and placed in drying room for four weeks. Bioactive compound of *P. grandiflora* were

extracted using methanol, hot water (distilled water), chloroform and acetone. For hot water extract the stock solution was prepared by adding 100 g of tubers powder in 1 L of distilled water then boiled for 15 minutes and followed by cooling and kept at 4^{o}C. For methanol, chloroform and acetone extraction 100 g of dried tuber will be added into 1 L of each solvent and allowed to homogenize for 24 hours at room temperature. All mixtures were filtered through Whatman filter papers and proper actions were taken to enssure that potential active constituents are not lost, distorted or destroyed during the preparation of the extracts from plant samples. Filtrates were concentrated using rotary evaporators (Rota vapor-R, Buchi, Switzerland). Different temperatures were used to evaporate extract solvents; acetone at 50°C, methanol at 60°C and chloroform at 59°C. Hot water extract was concentrated using freeze dryer. All concentrated sample were further dried into powder at room temperature.

4.3.3 Qualitative phytochemical analysis

A stock solution of 2 mg/ml was prepared by dissolving 40 mg of crude dried extract in 20 ml of their extraction solvent.

4.3.3.1 Test for phenol and tannins

Crude extract was mixed with 2 ml of 2% solution of $FeCl_3$ (ferric chloride). A brown, red or black coloration indicate presence of phenols and tannins.

4.3.3.2 Test for flavonoids

A stock solution of 1 ml of each crude plant extracts was poured into a test tube, followed by adding few drops of diluted NaOH (4mg/ml) solution and formation of intense yellow colour appear, then turns into colorless on addition of few drops of 32% HCl indicating the presence of flavonoids.

4.3.3.3 Determination of saponins

Froth test was used to determine the presence of saponins in the crude extract, where 1 ml of crude extract solution was placed into a test tube containing 5 ml distilled water and shaken vigorously, then left to stand for 10 min. The formation of stable foam was taken as an indication for the presence of saponins.

4.3.3.4 Determination of cardiac glycosides

The presence of glycosides in the crude extracts was determined by adding 4 ml of plant extract and 2 ml of 96% of glacial acetic acid having a single drop of ferric chloride solution. The sample mixture was underplayed with 1ml of concentrated sulphuric acid. The presence of glycoside was confirmed by the formation of a brown ring at the interface.

4.3.3.5 Test for steroids

The crude extract was mixed with acetic acid and few drops of sulfuric acid and produced a green or violet color indicating the presence of steroids.

4.3.3.6 Test for terpenoids

Crude extract of 5 ml were mixed with 2 ml of chloroform, followed by 3 ml of concentrated sulphuric acid. The resulting reddish-brown color indicated the presence of terpenoids in the plant extract.

4.3.3.7 Test for alkaloids

The presence of alkaloids in *P. grandiflora* crude extract was determined using Dragendoff test, where 1ml of the crude extract was warmed with 2% sulphuric acid and few drops of Dragendorff reagent was added. The presence of alkaloids was determined by formation of reddish brown precipitate in the mixture.

4.3.3.8 Test for coumarins

The presences of coumarins was determined by mixing 3 ml of NaOH with 2 ml of crude extract. Formation of yellow color indicated the presence of coumarins.

4.3.3.9 Test for resins

Few drops of acetic anhydride solution were added in 1 ml of crude extract then 1 ml of concentrated sulphuric acid was added. Resins give coloration ranging from orange to yellow.

4.3.4. Quantitative phytochemical evaluation

4.3.4.1 Total phenolic content

The total phenolic content of the plant extract was determined according to Folin-Ciocalteu (FC) method as previously explained by Stankovic, (2011) with slight modification. A stock solution of 1 mg/ml of crude extracts were prepared, then 20 µl of each extracts were poured in triplicate into 96 well plate containing 80 µl of dH_2O and 20 µl of 10% FC reagent was added to the diluted sample and allowed to stand for 1 min before 60 µl of 7% sodium carbonate (Na_2Co_3) was added to the mixture. To stop the reaction, 120 µl of dH_2O was added to the mixture for further dilution and the mixture read in a VersaMax Microplate Reader (Molecular Devices, Sunnyvale, USA) after 30 min @ 760 nm. The same procedure was repeated for gallic acid and calibration curve was constructed using linear regression. The concentration of phenolics was read in mg/ml based on the absorbance and gallic acid equivalent for the content (mg GA/g of the extract).

4.3.4.2 Total flavonoids content

The total flavonoid content was determined using method reported by Olajuyigbe and Afolayan, 2011 with slight modification. All tests were carried out in triplicate. A working concentration of 1

mg/ml of crude extract was prepared and 100 µl of 2% aluminum chloride ($AlCl_3$) was added to 100 µl of the crude extract in a 96 well plate and allowed to stand at room temperature for 60 min. Absorbance was measured at 420 nm using a VersaMax Microplate Reader (Molecular Devices, Sunnyvale, USA). The same procedure was repeated for quercetin and calibration curve was constructed using linear regression. The concentrations of flavonoid were read in (mg/ml) based on the absorbance and total flavonoid concentrations were expressed as quercetin equivalent per gram of the extract (mg QE/g).

4.3.5 Antioxidant activity

The free radical scavenging capacity of the extracts was determined using DPPH (2, 2-diphenyl1-picrylhydrazyl) radical as described by du Toit *et al*., (2001) with minor alterations. A concentration of 1 mg/ml of the crude extracts and 125 mM of DPPH/ethanol solution were prepared. About 100 µl of crude extracts were added in 96 well microplates containing 100µl of distilled water in triplicate. Serial dilution was made and 200µl of DPPH/ethanol was added to each well containing the mixture. The extent of reduction of the DPPH radical was determined by measuring the absorption at 517nm using VersaMax Microplate Reader (Molecular Devices, Sunnyvale, USA). Ascorbic acid was used as a reference standard and DPPH solution was used as the control. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the following equation:

$$\mathrm{RSA}(\%) = \left[\frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}}\right] \times 100\%$$

The scavenging activity was plotted against concentration and IC_{50} (the extract concentration providing 50% of radicals scavenging activity) value was calculated from the graph by linear regression analysis.

4.3.6 Thin layer chromatography

All dried extract were prepared at a concentration of 10 mg/ml with their extraction solvents. Different developing solvent systems with different polarity were prepared as EMW (ethyl acetate: methanol: water) 10:1.35:1 (polar/neutral), BEA (benzene: ethanol: ammonia) 18:2:0.2 (non-polar/basic), and CEF (chloroform: ethyl acetate: formic acid) 10:8:2 (Intermediate polarity/acidic). TLC analysis plate with Silica gel 60 F254 TLC (Merck, Germany), 10X10 cm was cut with ordinary household scissors. Plate markings were made with soft pencil and a ruler. Glass capillaries were used to spot the extracted sample for TLC applied sample volume 2 µl of the sample by using capillary at distance of 1 cm at 3 track. TLC chambers were filled with each developing solvent and TLC plates were put inside then close the chambers with the lids. The solvent was left to run. After running the experiment, the TLC plates were removed and solvent fronts were marked immediately with a pencil. The plates were dried at room temperature and the separated samples were visualized by spraying with vanillin-sulphuric acid (0.1 g vanillin: 28 ml methanol: 1 ml sulphuric acid) solution and heat TLC plates in the oven at 110^{o}C for optimal color development. The developed spots were circled immediately and the R_f values were calculated using the following equation:

$$R_f = \frac{\text{Distance traveled by the solute}}{\text{Distance traveled by the solvent front TLC plates}}$$

4.3.7 Bioautography

Ten µl (10 mg/ml) of each extract was loaded onto TLC plates in a narrow band and eluted using the three different mobile solvent systems (CEF, BEA and EMW). The developed plates were dried under a stream of fast moving air for six days to remove traces of solvent on the plates. Overnight cultures for *E. coli*, *K. pneumonia* and *S. aureus* of 0.5 MacFarland standard in Mueller-

Hinton broth were prepared. The prepared chromatograms were sprayed with the bacterial suspension until wet. This process was carried out in a Laminar flow cabinet (Labotec, SA). Thereafter, the plates were incubated overnight at 37°C and 100% relative humidity in the dark and then sprayed with a 2 mg/ml solution of p-iodonitrotetrazolium violet (Sigma, USA) (INT) (Begue and Klein, 1972) and further incubated overnight. White bands indicate where reduction of INT to the colored formazan did not take place due to the presence of compounds that inhibited the growth of tested organisms.

4.3.8 Infra-red spectroscopy

Infra-red spectra of the crude extract of *P. grandiflora* tubers were recorded to detect various functional groups responsible for biological activities. Perfectly, about 10 mg of dried powder of the extracts were placed on the sample chamber of FTIR spectrophotometer and the spectra were recorded in the scan range of 4000-400 cm^{-1} with a resolution of 4 cm^{-1} on Nicolet Avatar 330 FTIR spectrometer. The FTIR spectroscopic studies revealed different characteristic peak values with various functional compounds in the extracts. Important absorption frequencies appeared in functional group region as well as fingerprint region of the spectra were noted down.

4.3.8 Statistical analysis

All the *in vitro* experimental results were presented as the mean of three parallel measurements and data were evaluated using student's t-test. P-values of 0.05 were regarded as significant. Results were processed by Microsoft Excel (2016).

4.4 RESULTS

4.4.1 Plant extraction

Extraction of *P. grandiflora* was successfully achieved using various solvent including methanol, acetone, chloroform and boiling water. The yield of sequential extracts (g) is shown in (**Table 4.1**). The amount obtained from boiling water, methanol, acetone and chloroform extracts are 8.909 g, 27.831 g, 3.456 g and 0.9229 g respectively.

Table 4.1: Extractive values of different solvent extracts of *P. grandiflora* tubers.

S.No	Solvents	Colour of extracts	Yield of the extract (in g)	Yield % (% w/w)
1	Boiling water	Dark brown	8.909	8.9
2	Methanol	Dark red	27.831	27.8
3	Acetone	Dark red	3.456	3.5
4	Chloroform	Light brown	0.9229	0.9

4.4.2 Qualitative phytochemical analysis

Phytochemical screening of *P. grandiflora* revealed the presence of various bioactive components which includes phenolics, saponins, alkaloids, tannins, Steroids, terpenoids, and flavonoids. Phenolics, terpenoids and alkaloids were present in all extract. These are the most prominent components; the result of preliminary phytochemical analysis of *P. grandiflora* tuber extract is presented in (**Table 4.2**). Coumarins, resins, and glycosides were not detected in all extract.

Table 4.2: Qualitative Phytochemical analysis of the *P. grandiflora* tubers extracted using different solvents.

Phytochemicals	Methanol Extract	Water Extract	Acetone Extract	Chloroform Extract
Phenol/Tannin	+++	+++	+++	++
Flavonoids	-	+++	-	+++
Saponins	+++	++	-	-
Glycoside	-	-	-	-
Steroids	+	-	+	+++
Terpenoids	+++	++	+++	+++
Alkaloids	+++	++	+++	++
Coumarins	-	-	-	-
Resins	-	-	-	-

Key: absent (-), slightly positive (+), Positive (++) and highly positive (+++).

4.4.3 Quantitative Phytochemical evaluation

4.4.3.1 Total phenolic content

The total phenolic contents was examined in the *P. grandiflora* tuber extracts using the Folin-Ciocalteu's reagent which is expressed in terms of standard gallic acid equivalent with the standard curve equation: y = 0.0078x – 0.608, r2 = 0.9984 (**Figure 4.1**). The values obtained for the concentration of total phenols are expressed as mg of GA/g of extract (**Table 4.3**). The total phenolic contents in the examined extracts ranged from 14.167 to 19.02 mg GA/g. The highest concentration of phenols was found in acetone extracts. Methanol extract showed to contain a lower concentration of phenolic.

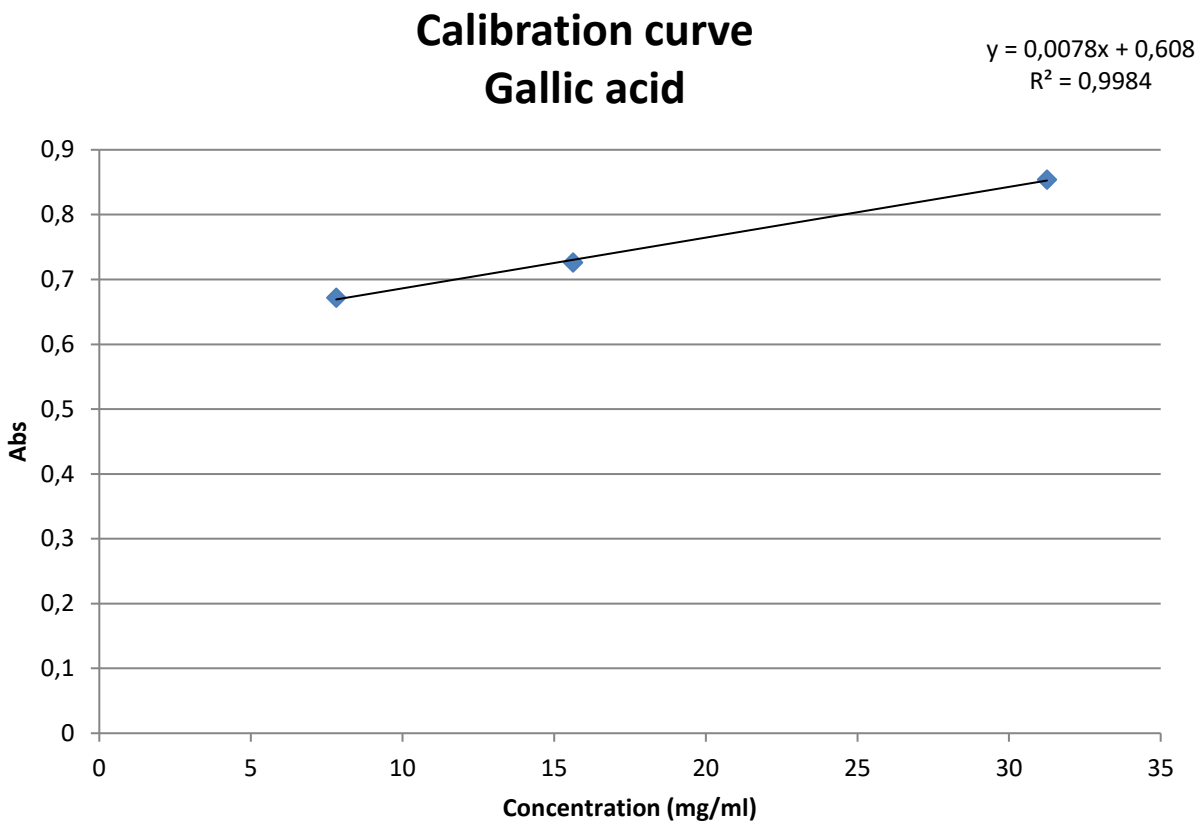


Figure 4.1: Calibration curve (Gallic acid).

Table 4.3: Total phenolic contents in the plant extracts expressed in terms of gallic acid equivalent (mg of GA/g of extract).

Extraction solvents	mg of GA/g of extracts
Boiling water	16.709±0.483
Acetone	19.02±0.334
Methanol	14.167±0.466

Each value is the average of three analyses ± standard deviation.

4.4.3.2 Total Flavonoids content

The total Flavonoids content in the examined *P. grandiflora* tubers extracts was determined using the spectrophotometric method with aluminum chloride. The content of flavonoids was expressed in terms of quercetin equivalent with the standard curve equation: y = 0.0229x – 0.035, r2 = 0.9778 (**Figure 4.2**). The values obtained for the concentration of total flavonoids are expressed as mg of QE/g of extracts (**Table 4.4**). The total flavonoids content in the examined extracts ranged from 26.603 to 34.621 mg GA/g. The highest concentration of flavonoids was measured in acetone extracts. Water extract showed to contain smaller concentration of flavonoids.

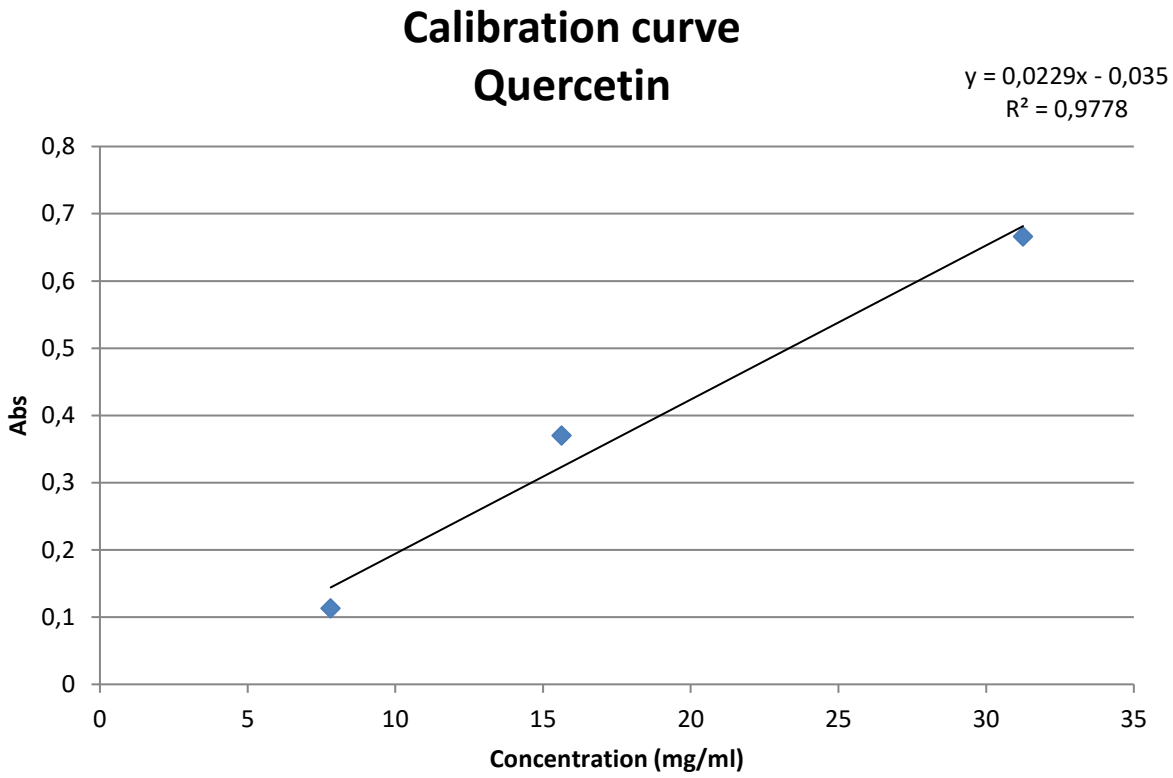


Figure 4.2: Calibration curve (Quercetins).

Table 4.4: Concentrations of flavonoids in the plant extracts expressed in terms of equivalent (mg of QE/g of extract)

Extraction solvents	mg of QE/g of extract
Boiling water	26.603±0.201
Acetone	34.621±0.309
Methanol	30.163±0.148

Each value is the average of three analyses ± standard deviation.

The amounts of TPC and TFC in *P. grandiflora* tubers extract is shown in **Figure 4.3**. Amongst all examined extracts flavonoids was presence in large quantity compared to phenolics content. Acetone was observed to possess highest flavonoids content with a value of 34.621 mg of QE/g whereas lowest value was observed in total phenolic content in methanol extract with a value of 14.167 mg of GA/g.

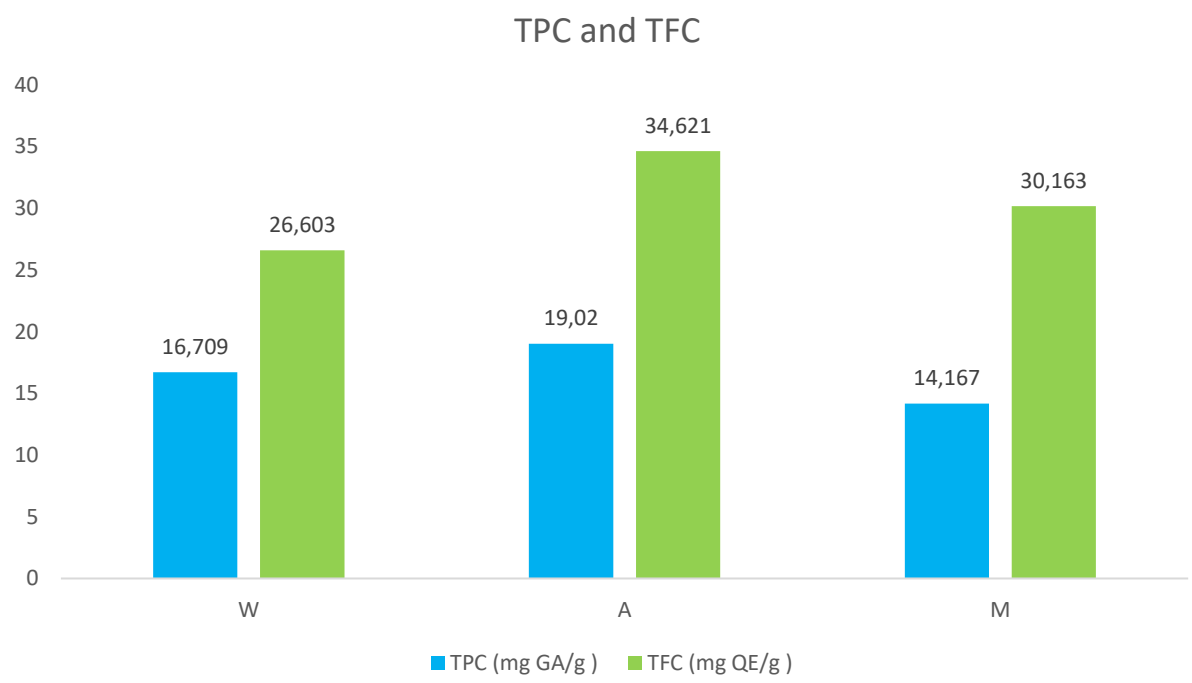


Figure 4.3: Total phenolic content (TPC) and total flavonoid content (TFC) of *P.grandiflora* tuber extracts, where, W= water extract, A= acetone extract and M= methanol extract.

4.4.4 DPPH radical scavenging

The radical scavenging activity and IC_{50} values of different extracts of *P.grandiflora* are shown in **Figure 4.4**. The concentration used in this study range from 1.3 mg/ml to 167 mg/ml. Normally, the higher percentage of radical scavenging activity and lower IC_{50} values indicate a higher antioxidant activity. Among the eight different concentrations used in the study 1.3 mg/ml has highest RSA value in all extract. Acetone extract revealed highest scavenging activity 85%, methanol extract 84% and water extract 86% whereas ascorbic acid at the same concentration showed in water extract (86%) (**Figure 4.4**). Water extracts exhibited an excellent radical scavenging activity with IC_{50} values 61.92 mg/ml. On the other hand, water extract displayed very weak antioxidant behavior with an IC_{50} value 106.65 mg/ml.

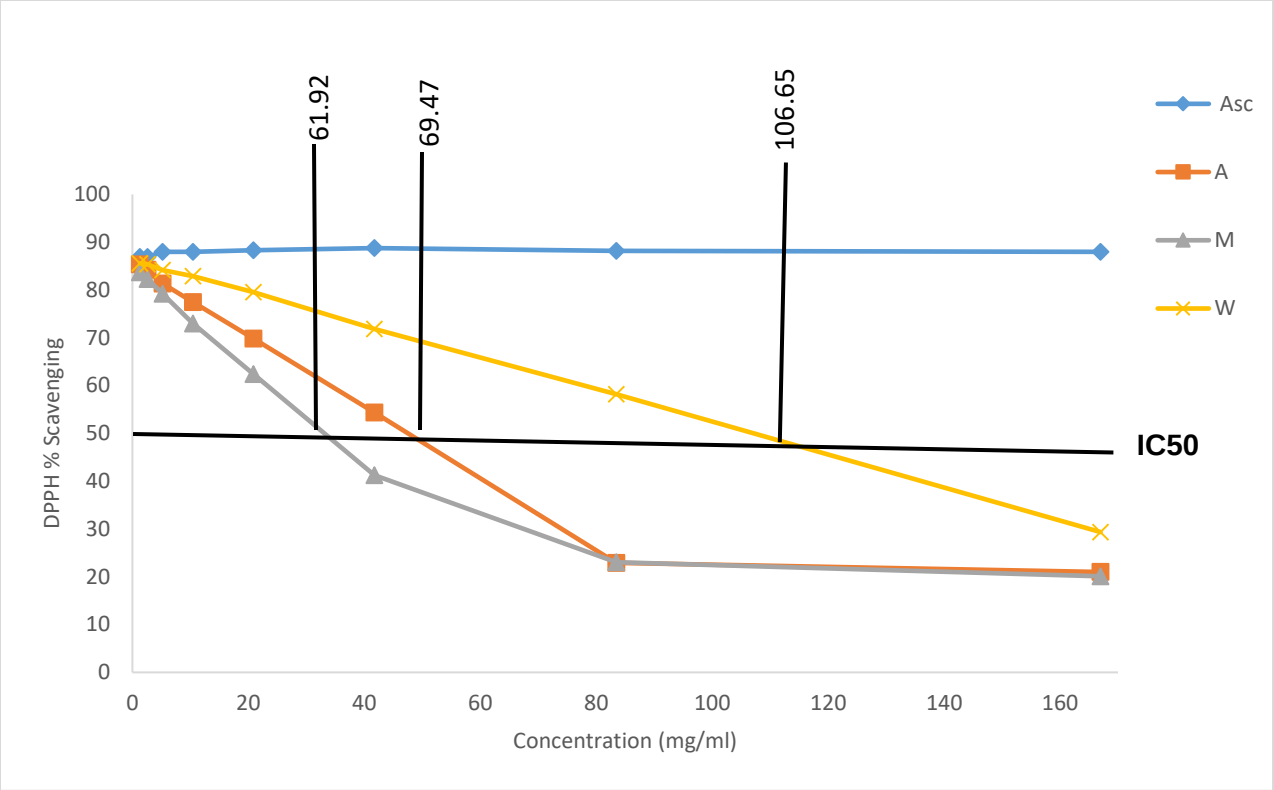


Figure 4.4: Antioxidant (DPPH scavenging) activity of investigated *P. grandiflora* tuber extracts presented as percentage of DPPH radicals scavenging and IC_{50} values (mg/ml).

4.4.5 Thin Layer Chromatography

TLC analysis also confirmed the presence of different kinds of phytochemicals in *P.grandiflora* tuber extracts. **Table 4.5** reports the R_f values for acetone, methanol and water extracts on three different developing solvent (BEA, CEF and EMW). **Figure 4.5** shows photographs of the studied TLC slides. Acetone extract reports more than three spots for various phytochemicals in all different polarity of developing solvent. The reported spots are separated with enough space and having various R_f values showing the presence of at least more than three phytochemicals in acetone extracts. Water extracts showed the maximum of two R_f values whereas methanol extract shows the maximum of three isolates.

Table 4.5: Retardation factor values of various extracts of *P. grandiflora* tuber extracts.

Extraction solvents	R_f Values		
	BEA (non-polar, basic)	EMW (polar, neutral)	CEF (intermediate polarity, acidic)
Water	0.097	0.232 0.623	0.147 0.471
Methanol	0.833 0.236	0.232 0.536 0.623	0.368 0.544
Acetone	0.139 0.236 0.347 0.514 0.917	0.362 0.580 0.623 0.869	0.221 0.353 0.544 0.794 0.912

Key: EMB (ethyl acetate: methanol: water) 10:1.35 (polar,neutral), BEA (benzene: ethanol: ammonia) 18:2:0.2 (non-polar/basic), and CEF (chloroform: ethyl acetate: formic acid) 10:8:2 (Intermediate polarity/acidic).

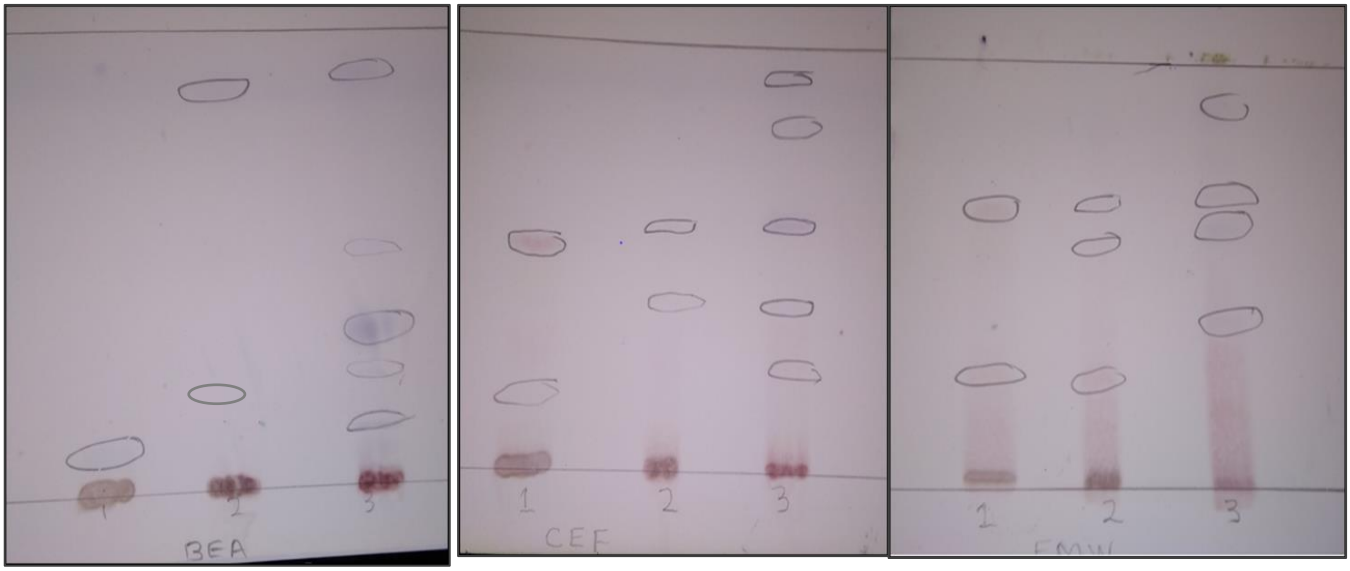


Figure 4.5: Photographs of TLC plates showing isolated extracts of *P. grandiflora* tuber extracts on three different solvents (BEA, CEF and EMW), where 1, 2 and 3 represent water extract, methanol extract, and acetone extract respectively.

4.4.6 Bioautography

To obtain some information on the active components, acetone, methanol and water extracts were analyzed by TLC on silica gel. The bioautography assay for qualitative antibacterial activity detection demonstrated well-defined inhibition zones against *E. coli* and *K. pneumonia* in BEA developing system with the R_f value of 0.236 corresponding to the TLC plate in **Figure 4.4**. Another growth inhibition was observed in CEF system in acetone extract with the R_f values of 0.353, 0.544 and 0.794 against *E. coli* (**Figure 4.6**). Most of the extract isolated compounds showed no active bands. In BEA system water and acetone extracts showed no activity of the isolated bands against all tested bacteria. Lack of bands separation was well-observed EMW system resulting in one large active band against all tested bacteria with water and acetone extract

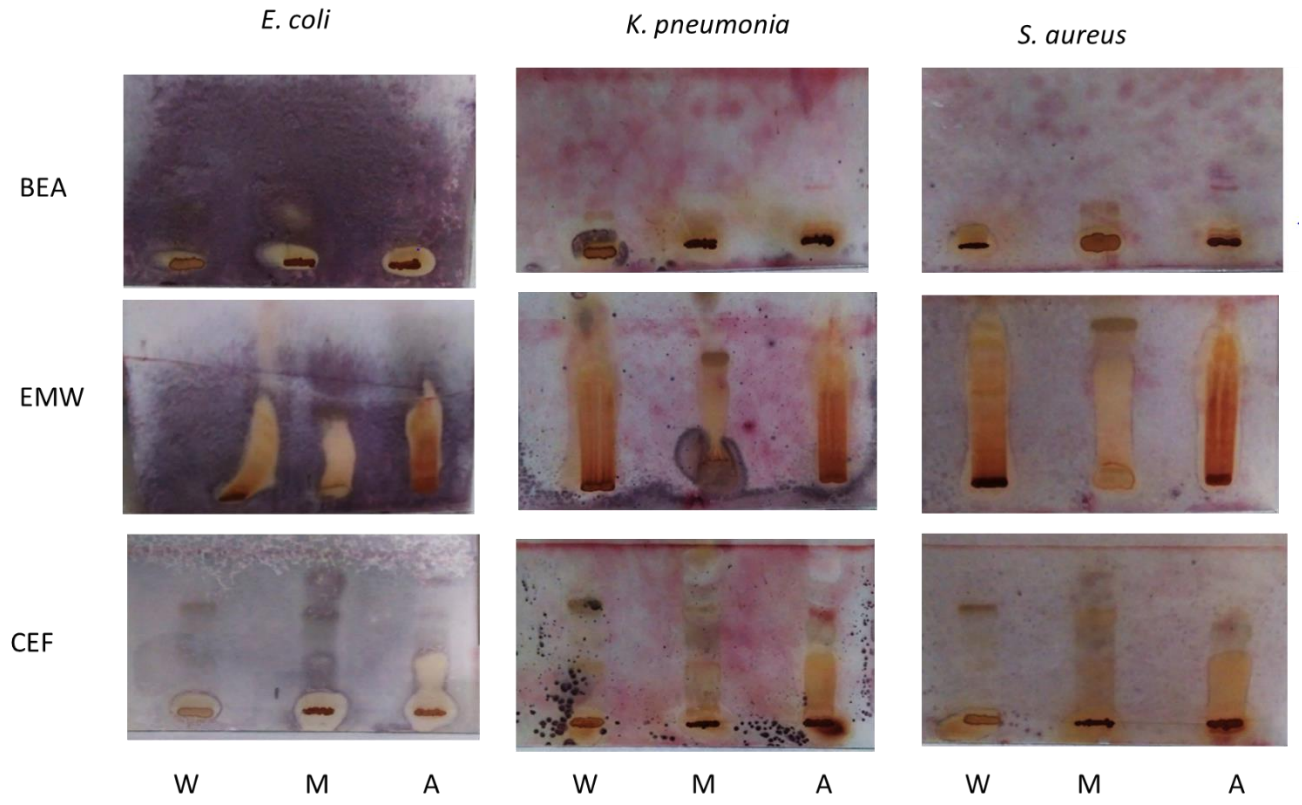


Figure 4.6: Inhibition of bacterial growth on bioautographic TLC plates of *P. grandiflora* tuber extracts against *E. coli*, *K. pneumonia* and *S. aureus*.

4.4.7 Infra-red spectroscopy analysis

The FTIR spectrum of *P. grandiflora* tuber extracts (prepared in different solvents) are given in **Figure 4.7**. The data on the peak values and the probable functional groups (obtained by FTIR analysis). All extract revealed the presence of Hydroxyl group (OH), C-H stretching and C=C carboxyl ranging from 3306-3153, 2923-2894 and 1689-1557 respectively.

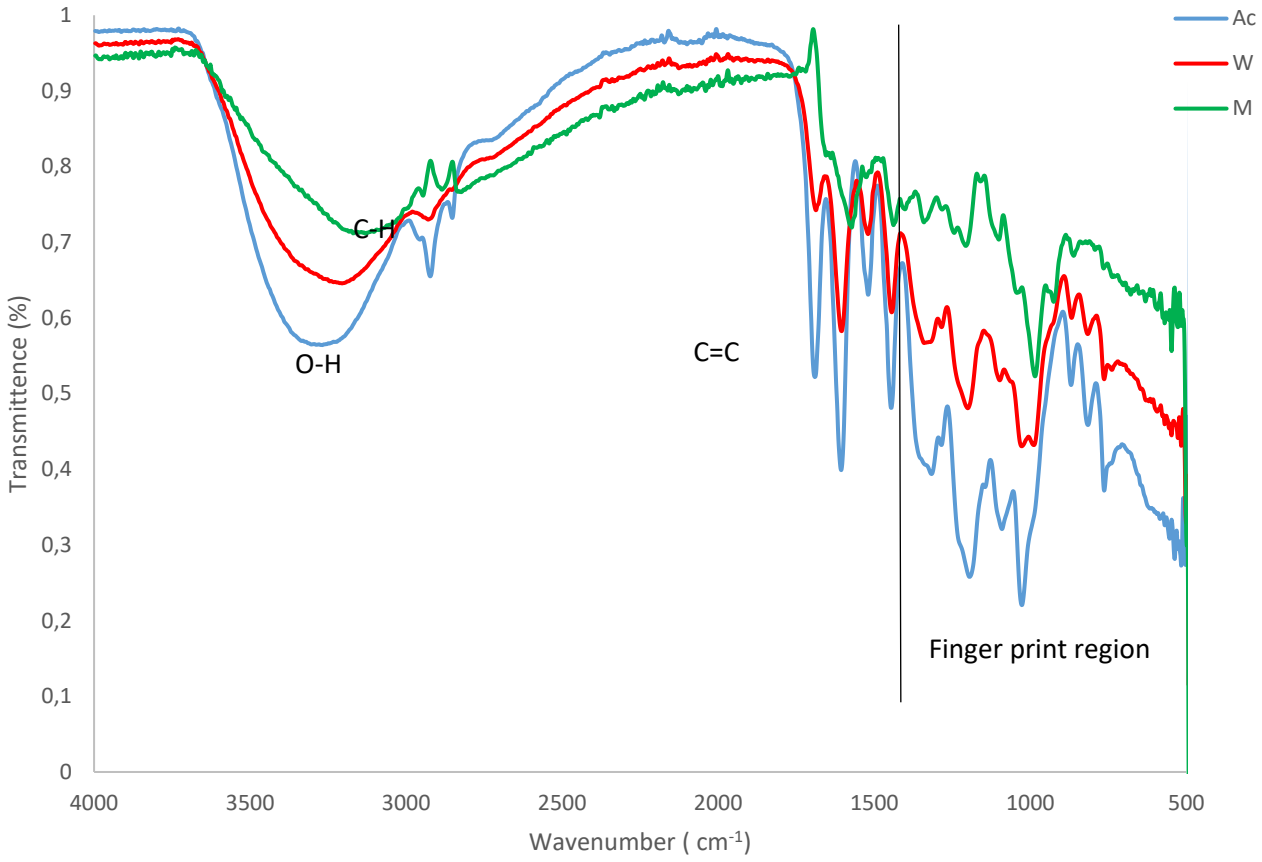


Figure 4.7: FTIR spectrum of *P. grandiflora* tubers extract prepared using different solvent where: Ac= acetone extract, W= water extract, and M= methanol extract.

4.5 **DISCUSSION**

The traditional ways of preparing most plants for medicinal purposes involve boiling in water to yield a decoction or soaking plant material in freshly boiled water to produce an infusion (McGaw *et al*., 2013). In this study, boiling water was used in order to mimic as closely as possible the traditional way of preparing *P. grandiflora*. However, hydrophilic compounds polar solvents; methanol and acetone were also used for extraction since they are known to be more active than lipophilic solvents (Złotek *et al*., 2016). Methanol extract was found to yield more crude extract compared to water and acetone extract. Chloroform is a non-polar solvent and has yielded the smallest crude extract. Hence, Non-polar solvent are known to produce small amount of crude extracts (Loyao *et al*., 2018).

It is interesting to note that the extracts of *P.grandiflora* revealed the presence of phenolic, saponins, alkaloids, tannin, steroids, terpenoids and flavonoids. Some of these bio-active ingredients have been reported in *Pyrenacantha staudtii*, which is the mostly studied species of genus *Pyrenacantha* (Odugbemi and Ayoola, 2008). Saponins as well as tannins are well known as antimicrobial and antioxidant active classes of bioactive phytochemicals in the plants. Similarly, this study provides scientific validation for usage of the *P. grandiflora* in folk medicine in our region as it contains those phytochemicals.

The total flavonoid and phenolic content of *P. grandiflora* were further quantified. However, different values of total phenolic and flavonoid content were observed with different solvents from the same plant part. Flavonoids content was higher compared to phenolic content with the highest value of 34.621 mg of QE/g with acetone extract. This confirms that solvent used for extraction played a role in the total flavonoids and phenolic content of plants extracts (Malualem *et al.*, 2011). It has been well established that flavonoids in nature are potential antioxidants. In addition,

quercetin which is a flavonoid that exists in numerous plants, possesses a very good antioxidant activity (Ogunmoyole *et al*., 2013).

An antioxidant is considered to be "any substance that, when present at low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate". In this study we found that the *P. grandiflora* tuber extracts have highest radical scavenging activity of 86% in water extract at the lowest concentration of 1.3 µg/ml and IC_{50} value of 61.92 mg/ml. This values were very close to those of ascorbic acid which was used as positive control. Generally, the antioxidant present in the body normally scavenge the free radicals produced and prevent the damage caused by them. Therefore these *P. grandiflora* tuber extracts can be of more important in food processing and pharmaceutical industries.

Methanolic and acetone extracts from *Marrubium peregrinum* was reported to have high concentration of total phenols and flavonoids which was in correlation with intense antioxidant activity of these extracts (Stankovic, 2011). Antioxidants greatly reduce the harm due to oxidants by the free radicals before they can attack the cells and prevent damage to lipids, proteins, enzymes, carbohydrates and DNA (Nimse and Pal, 2015), so further knowledge on cytotoxicity of *P. grandiflora* tubers is needed.

An important factor in quantifying the movement of a compound on a stationary phase e.g. silica with a certain solvent system is the retardation factor (R_f) value and is the ratio of the distance moved by the compound from its origin due to the movement of the solvent (Wellawatta *et al*., 2017). Due to the difference in polarity of the different solvent systems, compounds had relatively high R_f values in polar solvents (Eloff *et al*., 2018). Hence, *P. grandiflora* tuber extracts had low R_f values in non-polar solvents like BEA. By using EMW, BEA and CEF as developing solvents, compounds with a wide range of polarities can be separated. Because the R_f value is constant for the same compound under defined conditions, the presence of clear bands with the same R_f value may mean that the same compounds are probably responsible for the antimicrobial activity

in the same extract tested against different microorganisms. This would suggest non-selective antimicrobial activity (Eloff et al., 2018).

Antimicrobial compounds present in different extracts were separated with different solvent systems and tested against *E. coli*, *S. aureus* and *K. pneumonia* with bioautography. The appearance of white areas against a purple-red background on the chromatograms as observed and this indicate inhibition of growth of the bacteria due to the presence of compound(s) that inhibit their growth. Actively growing microorganisms have the ability to reduce INT to a purple-red color (Begue and Klein, 1972). In the presence of active plant compounds on the chromatograms, the growth of the organism is inhibited. In some cases, no inhibition of microbial growth was observed. The absence of activity could be due to evaporation of the active compounds, photo-oxidation or due to the very little amount of the active compounds (Masoko *et al*., 2005). All the compounds with activity based on bioautography in future studies can be isolated and identified.

The overall bioautogaphy results agreed with previously published results (Masoko *et al*., 2005) that the substances responsible for the antimicrobial activity were mainly non-polar in nature. Some study shows that acetone and methanol extracts of *Punica granatum* and *Delonix regia* have good activity against methicillin-resistant *S. aureus* (Aqil *et al*., 2005). It is important to realize that bioautography is not a quantitative measure of antimicrobial activity. It only indicates the number of compounds that were separated with antimicrobial activity (Eloff *et al*., 2018).

In any bio-organic molecule, its functional groups influence its biological activity and they contribute significantly to their inherent acid–base properties, solubility, crystal structure and stereochemistry, all these properties are supposed to influence the absorption, distribution, metabolic extraction, and toxicity of bioactive molecules (Kim and Rajapakse, 2005). Hence, functional groups analysis plays a vital role in understanding the overall physicochemical

properties of the extract. Also, identification of the functional groups helps to evaluate their structure activity relationships.

In the present work, FTIR spectral analysis of the tubers of *P. grandiflora* showed the presence of phytochemicals carrying hydrogen bonded –OH functional group. It is well established that hydroxyl functional group is an integral part of most of the phenolic phytochemicals such as flavonoids and tannins (Ananga *et al*., 2017). Recent studies show that several plant products, including polyphenolic substances (e.g., flavonoids and tannins) and various herbal extracts, show antioxidant and anti-inflammatory activities (Dzoyem and Eloff, 2015). Our results indicate that the *P. grandiflora* tubers contain various biologically active functional groups, viz. alcoholic, ester, aldehydes etc., hence we can confirm that the plant possesses bioactive phytochemicals.

4.6 CONCLUSION

It is trusted that the present study would direct to the establishment of some compounds that could be used to research new and more potent antimicrobials and antioxidants of the plant origin. These phytochemical screening of tubers of *P. grandiflora* certainly encourages future advanced research activities on chromatographic isolation of these compounds in their pure state using either HPLC, column chromatography or gas chromatography and furthermore to evaluate in detail the *in vivo* biological activities of such isolated compounds. In addition to being effective against bacteria, these compounds could exhibit inhibitory effects against viruses and parasites.

4.7 *REFERENCE*

Ananga, A., Obuya, J., Ochieng, J. and Tsolova, V., (2017). Grape Seed Nutraceuticals for Disease Prevention: Current Status and Future Prospects. In Phenolic Compounds-Biological Activity. *InTec*h.

Aqil, F., Khan, M.S.A., Owais, M. and Ahmad, I., (2005). Effect of certain bioactive plant extracts on clinical isolates of *β*-lactamase producing methicillin resistant *Staphylococcus aureus*. *Journal of Basic Microbiology*, 45(2), pp.106-114.

Ashbolt, N.J., (2004). Risk analysis of drinking water microbial contamination versus disinfection by-products (DBPs). *Toxicology*, 198(1-3), pp.255-262.

Atindehou, K.K., Kone, M., Terreaux, C., Traore, D., Hostettmann, K. and Dosso, M., (2002). Evaluation of the antimicrobial potential of medicinal plants from the Ivory Coast. *Phytotherapy Research*, 16(5), pp.497-502.

Begue, W.J., Klein, R.M. (1972). The use of tetrazolium salts in bioautographic procedure. *Journal of Chromatography* 88: 182-184.

du Toit, R., Volsteedt, Y. and Apostolides, Z., (2001). Comparison of the antioxidant content of fruits, vegetables and teas measured as vitamin C equivalents. *Toxicology*, 166(1-2), pp.63-69.

Dzoyem, J.P. and Eloff, J.N., (2015). Anti-inflammatory, anticholinesterase and antioxidant activity of leaf extracts of twelve plants used traditionally to alleviate pain and inflammation in South Africa. *Journal of Ethnopharmacology*, 160, pp.194-201.

Eloff, J.N., Angeh, I.E. and McGaw, L.J., (2018). Solvent-solvent fractionation can increase the antifungal activity of a *Melianthus comosus* (Melianthaceae) acetone extract to yield a potentially useful commercial antifungal product. *Industrial Crops and Products*, 111, pp.69-77.

Eloff, J.N., Katerere, D.R. and McGaw, L.J., (2008). The biological activity and chemistry of the southern African Combretaceae. *Journal of Ethnopharmacology*, 119(3), pp.686-699.

Faidallah, H.M., Panda, S.S., Serrano, J.C., Girgis, A.S., Khan, K.A., Alamry, K.A., Therathanakorn, T., Meyers, M.J., Sverdrup, F.M., Eickhoff, C.S. and Getchell, S.G., (2016). Synthesis, antimalarial properties and 2D-QSAR studies of novel triazole-quinine conjugates. *Bioorganic & medicinal chemistry*, 24(16), pp.3527-3539.

Haque, M.E., Asanuma, M., Higashi, Y., Miyazaki, I., Tanaka, K.I. and Ogawa, N., (2003). Overexpression of Cu–Zn superoxide dismutase protects neuroblastoma cells against dopamine cytotoxicity accompanied by increase in their glutathione level. *Neuroscience research*, 47(1), pp.31-37.

Kim, S.K. and Rajapakse, N., (2005). Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review. *Carbohydrate polymers*, 62(4), pp.357-368.

Loyao, A.S., Villasica, S.L.G., Peña, P.L.L.D. and Go, A.W., (2018). Extraction of lipids from spent coffee grounds with non-polar renewable solvents as alternative. *Industrial Crops and Products, 119, pp.152-161.*

Masoko, P., Picard, J. and Eloff, J.N., (2005). Antifungal activities of six south African *Terminalia* species (Combretaceae). *Journal of Ethnopharmacology*, 99(2), pp.301-308.

McGaw, L.J., Jäger, A.K. and Van Staden, J., (2000). Antibacterial, anthelmintic and anti-amoebic activity in South African medicinal plants. *Journal of ethnopharmacology*, 72(1-2), pp.247-263.

Nimse, S.B. and Pal, D., (2015). Free radicals, natural antioxidants, and their reaction mechanisms. *Rsc Advances*, 5(35), pp.27986-28006.

Odugbemi, T. and Ayoola, A., (2008). Medicinal plants from Nigeria: an overview. A Textbook of Medicinal Plants From Nigeria (ed. Tolu Odugbemi), University of Lagos Press, Akoka-Yaba, Lagos p, pp.9-17.

Ogunmoyole, T., Inaboya, S., Makun, J.O. and Kade, I.J., (2013). Differential antioxidant properties of ethanol and water soluble phytochemicals of false nutmeg (Monodora myristica) seeds. *International Journal of Biochemistry and Biotechnology*, 2(1).

Olajuyigbe, O.O. and Afolayan, A.J., (2011). Phenolic content and antioxidant property of the bark extracts of *Ziziphus mucronata* Willd. subsp. mucronata Willd. *BMC complementary and alternative medicine*, 11(1), p.130.

Prabakaran, P. and Ravindran, A.D., (2011). A comparative study on effective cell disruption methods for lipid extraction from microalgae. *Letters in applied microbiology*, 53(2), pp.150-154.

Rakelly de Oliveira, D., Relison Tintino, S., Morais Braga, M.F.B., Boligon, A.A., Linde Athayde, M., Douglas Melo Coutinho, H., de Menezes, I.R.A. and Fachinetto, R., (2015). In vitro antimicrobial and modulatory activity of the natural products silymarin and silibinin. *BioMed research international*.

Saha, S., Santosham, M., Hussain, M., Black, R.E. and Saha, S.K., (2018). Rotavirus Vaccine will Improve Child Survival by More than Just Preventing Diarrhea: Evidence from Bangladesh. *The American journal of tropical medicine and hygiene*, 98(2), pp.360-363.

Samie, A., Obi, C.L., Bessong, P.O. and Namrita, L., (2005). Activity profiles of fourteen selected medicinal plants from Rural Venda communities in South Africa against fifteen clinical bacterial species. *African journal of Biotechnology*, 4(12).

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K.M. and Latha, L.Y., (2011). Extraction, isolation and characterization of bioactive compounds from plants' extracts. African Journal of Traditional, *Complementary and Alternative Medicines*, 8(1).

Stankovic, L. (2011). Transforming trauma: A qualitative feasibility study of integrative restoration (iRest) Yoga Nidra on combat-related post-traumatic stress disorder. *International Journal of Yoga Therapy*, 21(1), 23–37.

Wellawatta, W.M.A.S., Munasinghe, M., Jeewandara, P. and Nawarathne, T.K., (2017), November. Comparative Phytochemical Screening and Chromatography Development to Determine the Suitability of Stem Parts of Justiciaadhatoda (Adhathoda) in the Preparation of Standardise Herbal Drugs. *In Proceedings of International Forestry and Environment Symposium* (Vol. 22).

Złotek, U., Mikulska, S., Nagajek, M. and Świeca, M., (2016). The effect of different solvents and number of extraction steps on the polyphenol content and antioxidant capacity of basil leaves (*Ocimum basilicum L*.) extracts*. Saudi journal of biological sciences*, 23(5), pp.628-633.

Zuma, T., Wight, D., Rochat, T. and Moshabela, M., (2016). The role of traditional health practitioners in Rural KwaZulu-Natal, South Africa: generic or mode specific?. BMC *complementary and alternative medicine*, 16(1), p.304.

CHAPTER FIVE: SYNTHESIS, CHARACTERIZATION AND *IN VITRO* ANTIBACTERIAL EVALUATION OF *PYRENACANTHA GRANDIFLORA* CONJUGATED SILVER NANOPARTICLES

5.1 SUMMARY

BACKGROUND: Amongst bacterial pathogens, antimicrobial resistance is a crisis that has been worsening over recent decades, resulting in serious and often fatal infections that cannot be treated by conventional means. Infections caused by common pathogenic bacterial strains, such as *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae*, take on a new significance once these microorganisms develop some form of antibiotic resistance. Some promising strategies to combat antibiotic resistance involve the use of traditional medicinal plants as well as metallic nanoparticles.

OBJECTIVE: This study aimed to evaluate the antibacterial activity of *Pyrenacantha grandiflora* Baill extracts when conjugated with silver nanoparticles against pathogenic bacteria.

METHODS: Silver nanoparticles were synthesized using the chemical and biological methods. Nanoparticles were analyzed with UV- Vis spectrophotometer, transmission electron microscopy (TEM) and energy dispersive X-ray analysis (EDX). Silver nanoparticles were conjugated to plant extracts and analyzed with Fourier transform infrared spectroscopy (FTIR). Well diffusion assay was used to evaluate the antimicrobial activity of the conjugated plant extracts and the microdilution method was ued to determine the minimum inhibitory concentrations (MIC). Minimum bacteriocidal concentrations were also determined.

RESULTS: The UV–Visible spectra of silver nanoparticles showed synthesis peak at 450 nm. FTIR analysis indicated functional bio-molecules associated with conjugated plant extracts formation. The nature of silver nanoparticles elemental composition was confirmed by their EDX diffraction. Transmission electron microscopy confirmed the synthesis of silver nanoparticles by

Magnetospirillum magnetotacticum bacteria characterized by spherical shape and mean particle size of 10 nm. Well diffusion assay showed that the activity of silver nanoparticles is improved with acetone axtract against all tested bacteria with the diameter range of 19-24 mm followed by silver nanoparticles conjugated methanol extract with diameter range of 13-18 mm. From the MIC results, the lowest MIC value of 0.0063 mg/ml was observed when biologically synthesized silver nanoparticle are conjugated with acetone and water extracts against methicillin-resistant *Staphylococcus aureus*. Chemically synthesized silver nanoparticles also showed lowest MIC value of 0.0063 mg/ml against *E. coli* when conjugated with acetone and methanol extracts. Similar activity was observed against *E. coli* and *K. pneumonia* respectively.

CONCLUSION: The *in-vitro* antibacterial activity of silver nanoparticles conjugated with *P. grandiflora* tuber extracts showed potential antibacterial property against multi-drug resistant pathogens such as MRSA, *Klebsiella pneumonia,* and *Escherichia coli.* The biosynthesized conjugates could be utilized as antimicrobial agents for effective disease management due to the synergistic antibacterial activity that was observed.

Keywords: Antibacterial activity, Silver nanoparticles, Plant extracts, Agar diffusion assay, Minimum bactericidal concentration.

5.2 INTRODUCTION

There is an urgent need for novel antibacterial agents. Therefore, research has focused on developing several strategies to address this issue. This has been done by trials in making novel antibacterial agents or artificially adjusting action of existing/momentarily effective antibiotics (Tillotson and Theriault, 2013). In the present scenario, medicinal plants and silver nanoparticles are emerging to address the challenge due to their efficacy as antimicrobial agents. A number of studies have proved that silver nanoparticles possess antimicrobial, anti-inflammatory, anti-angiogenic, anticancer and antiviral properties with several advantages such as less toxicity, enhanced biodegradability, and bioavailability even in industrial applications (Subbaiya *et al*., 2017; Barabadi *et al*., 2017; Saratale *et al*., 2017 and Ramkumar *et al*., 2017).

Plants and their derived products have long been used by humans for medicinal purposes. It is estimated today that about 80% of the world's population uses botanical preparations as medicines to cover their health needs (Mustafa *et al*., 2017). Thus, there are about half a million of therapeutic plants, far and wide; for the vast majority of them their medicinal activity had not been explored yet, although their restorative activities could be unequivocal in the treatment of present and future diseases (Rasool Hassan, 2012). Several studies have been done in South Africa to highlight antibacterial activities of some medicinal plants. For example, *Pyrenacantha grandiflora* Baill showed to have antibacterial activities (Ramalivhana , 2010).

To develop novel treatment, two or more effective treatment strategies can be combined. This study is based on the assumption that the co-existence of two or more different molecules with entirely different mode of actions in the form of hybrid molecule will produce a synergistic effect. Hybrid molecules thus, could offer advantages such as dosage compliance, minimizing toxicity and overcoming drug resistance when compared to the parent counterparts (Wang *et al*., 2007). In most studies, an increase in antimicrobial activity was reported after conjugation of antibiotic with the nanoparticles. Hence, AgNPs was reported to improve antimicrobial activity of ampicillin

(Abhishek and Hemlata, 2014). Silver nanoparticles were also reported to have antibacterial activity when conjugated with plant extracts (Yazdi *et al*., 2018). This study aimed to evaluate antibacterial the activity of *Pyrenacantha grandiflora* Baill extracts when conjugated with silver nanoparticles against problematic pathogenic bacteria (*Staphyloccocus aureus* (MRSA), *Escherichia coli, Klebsiella pneumoniae*).

5.3 MATERIALS AND METHODS

5.3.1 Used microorganism and growth conditions

The microorganisms that were used in this study include *Magnetospirillum magnetotacticum* MS-1 (American Type Culture Collection) which was grown in modified chemically defined growth medium supplemented with ISOGRO. Methicillin-resistant *Staphylococcus aureus* ATCC 25923, methicillin-susceptible *Staphylococcus aureus* ATCC 33594 were sub-cultured on mannitol salt agar, *Escherichia coli* ATCC 35218 and 25922 were sub-cultured on MacConkey agar, *Klebsiella pneumonia* ATCC 700603 was sub-cultured on nutrient agar (Rochelle, SA). An inoculum of each bacterial strain was suspended in 5 ml of Mueller Hinton broth (Rochelle, SA) and incubated for 3 hours at 37°C. The cultures were diluted with Mueller Hinton broth and adjusted to give a concentration of bacterial cells equivalent to a 0.5 McFarland standard prior to the antibacterial testing.

5.3.2 Chemical synthesis of silver nanoparticles

5.3.2.1 Silver nanoparticles preparation

Silver nanoparticles were prepared using Turkevich protocol (1951). Briefly, 1 mM of silver nitrate ($AgNO_3$) powder (Sigma, USA) and 1% tri-sodium citrate of analytical grade purity were used. The silver colloid was prepared by using chemical reduction method. A total of 100 ml of 1mM $AgNO_3$ was heated to boiling, to which 5ml of 1 % tri-sodium citrate was added drop by drop. During the process, the solution was mixed vigorously. The solution was heated until colour's change is evident (yellowish brown). Then it was removed from the heating plate and stirred until cooled to room temperature.

5.3.2.2 UV-Vis spectrum analysis

The characterization of silver nanoparticles were done using UV-Visible Spectrophotometer (Specord 210, Analytikjena spectrometer). The reduction of silver nitrate to silver nanoparticles by sodium citrate was confirmed by observing a broad absorbance peak between 400-500 nm.

5.3.2.3 High resolution- transmission electron microscopy

Further characterization was done using HR-TEM studies the sample was prepared by placing a drop of the nanoparticle solution onto a carbon-coated copper TEM grid. The sample was then dried under an infrared lamp for a period of 45 min for the solvent to evaporate. High-resolution TEM images were obtained on JEOL TEM model no 2100 instrument operated at an accelerating voltage of 200 kV and 0.23 nm resolution.

5.3.3 Biological synthesis of silver nanoparticles

5.3.3.1 Cultivation of *Magnetospirillum magnetotacticum* bacteria

Silver nanoparticles were synthesized using *Magnetospirillum magnetotacticum* MS-1. These bacteria were cultured in chemically defined growth media prepared with slight modification. ISOGRO was added in the media to enhance the growth of bacteria by shortening the lag phase of growth resulting in high bacterial yield. The source of silver was silver nitrate which was added as a source of metal in the growth media. The MS-1 cells were grown for 4 days at 30^{o}C in airtight 50 ml falcon tubes wrapped with parafilm because they grow well in micro-aerobic condition. Tubes were covered with foil to prevent photo degradation of silver nitrate in the media. Synthesis of silver nanoparticles was initially confirmed by color change in the media.

5.3.3.2 Analysis of silver nanoparticles synthesis by HR -TEM

High- Resolution Transmission Electron Microscopy (HR-TEM) was used to detect the whether silver nanoparticles were produced by *Magnetospirillum magnetotacticum* cells. The sample was prepared by placing a drop of the bacterial culture onto a carbon-coated copper TEM grid. The sample was then dried under an infrared lamp for a period of 45 min. High-resolution TEM images were obtained on JEOL TEM model no 2100 instrument operated at an accelerating voltage of 200 kV and 0.23 nm resolution.

5.3.3.3 Isolation of silver nanoparticles

The isolation of silver nanoparticles from *Magnetospirillum magnetotacticum* culture was done using MACS magnetic separation column (Miltenyi Biotec). Briefly, *M. magnetotacticum* bacterial

cells were suspended in 20 mM HEPES-4 mM EDTA (pH 7.4) and broken open by sonication for 5 min at 21°C. The unbroken cells were removed by centrifugation at 9000 rpm for 30 min. The supernatant was harvested and passed through MACS magnetic separation column following manufacturer's protocol. Unbound magnetic particles were washed using 10 mM HEPES-200 mM NaCl (pH 7.4) then the silver nanoparticles were eluted with 10 mM HEPES (pH 7.4).

5.3.4 Preparation of plant extracts conjugated with silver nanoparticles

Pyenacantha grandiflora extracts were extracted using acetone, methanol, and water as a solvents as explained in Chapter 4. Non-covalent modification of plants extract with silver nanoparticles was applied in the generation of hybrid molecules (Tom *et al.*, 2004). Synthesized silver nanoparticles from both chemical and biological methods were mixed with 10 mg/ml of each plant extracts (acetone, water, and methanol extracts) and incubated at 4^{o}C for 24 hours.

5.3.5 Characterization of the conjugates

All conjugates were analyzed using Fourier- transmission electron microscopy (FTIR) in a range of 400-4000 cm^{-1} to detect various functional groups formed after conjugation that are responsible for biological activities. A precise volume of about 500 µl of the conjugates were placed on the sample chamber of FTIR spectrophotometer and the spectra were recorded in the scan range of 400–4000 cm^{-1} with a resolution of 4 cm^{-1} in a Nicolet Avatar 330 FTIR spectrometer.

5.3.4 Well diffusion assay

The antibacterial activities of conjugates were initially determined by the well diffusion method. The zones of inhibition were recorded in millimeter (mm). Briefly, bacterial suspensions were prepared with the turbidity of 0.5 McFarland Standard. Mueller-Hinton agar plates were inoculated with *E. coli*, *K. pneumonia* and *S. aureus*. Wells with a diameter of 6 mm were cut using a cork borer and filled with 30 µl of the conjugates and reference samples (plant extract and silver nanoparticles). Distilled water was used as a negative control. Plates were incubated 24 hours at 37°C. After incubation, the growth inhibition zone diameters were measured.

5.3.5 Microdilution assay

The minimum dilution of all extracts plus conjugates samples that inhibits the growth of the microorganism were denoted as minimum inhibitory concentration (MIC) (Samie *et al.*, 2005). Distilled water was used as negative control and gentamycin as a positive control. After adding INT, the results were read by observing the color change and determining the MIC. All the conjugates that show activity (reduced or no color change) were inoculated again in the agar plate and incubated overnight to determine the minimum bactericidal concentration (MBC).

5.3.6 Fractional Inhibition Concentration Index (FICI) calculations

Determination of the mutual influence of *P. grandiflora* tuber extracts and silver nanoparticles in conjugates was done using fractional inhibition concentration index by the following formula:

$$FICI = \frac{MIC\ of\ AB}{MIC\ of\ A} + \frac{MIC\ of\ AB}{MIC\ of\ B}$$

Where AB represent a combination of *P. grandiflora* tubers extracts (A) and silver nanoparticles (B). Results were interpreted as synergy (FICI≤0.5), antagonism (FICI>4) and no interaction or additive (FICI>0.5-4.0).

5.4 RESULTS

5.4.1 Analysis of silver nanoparticles

Silver nanoparticles were successfully synthesized using the chemical method. The color of silver nitrate solution changed from colorless (**Figure 5.1A**) to yellow color (**Figure 5.1B**) when 1% of sodium citrate is added. This color originates from coherent electron motion in the colloidal solution, giving rise to a characteristic absorption of light at a wavelength of 400-500 nm to confirm the synthesis of nanoparticles.

Figure 5.1: Images illustrating the color change of silver nitrate from colorless (A) to yellow (B) when citrate was added to confirm the synthesis of silver nanoparticles.

5.4.1.1 UV-Visible analysis of silver nanoparticles

Ultra-violet visible spectroscopic studies showed the peak at 434 nm (maximum absorbance) which confirms the presence of silver nanoparticles as shown in peak 2 (**Figure 5.2**). Absorption peak 1, obtained at 290 nm (**Figure 5.2**) indicates the un-reacted silver nitrate in solution.

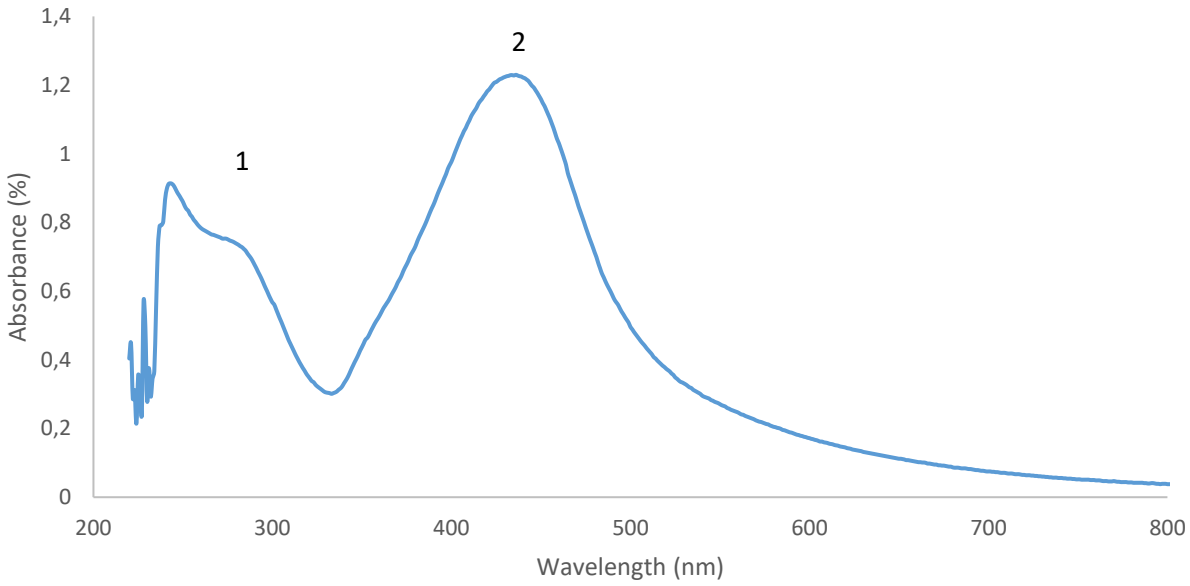


Figure 5.2: UV–Visible spectra of silver nanoparticles. Peak 1 represents the unreacted silver nitrate and peak 2 represents silver nanoparticles.

5.4.1.2 Transmission electron microscopy analysis of silver nanoparticles

The morphology of nanoparticles refer to the nanocubes shape. In the higher magnification image, silver nanoparticles of 5-33 nm size were also observed (**Figure 5.3 A**). However, the average size of the nanoparticles was found to be 13 nm. The EDX analysis confirmed that the particles were composed by elemental silver (**Figure 5.3 B**).

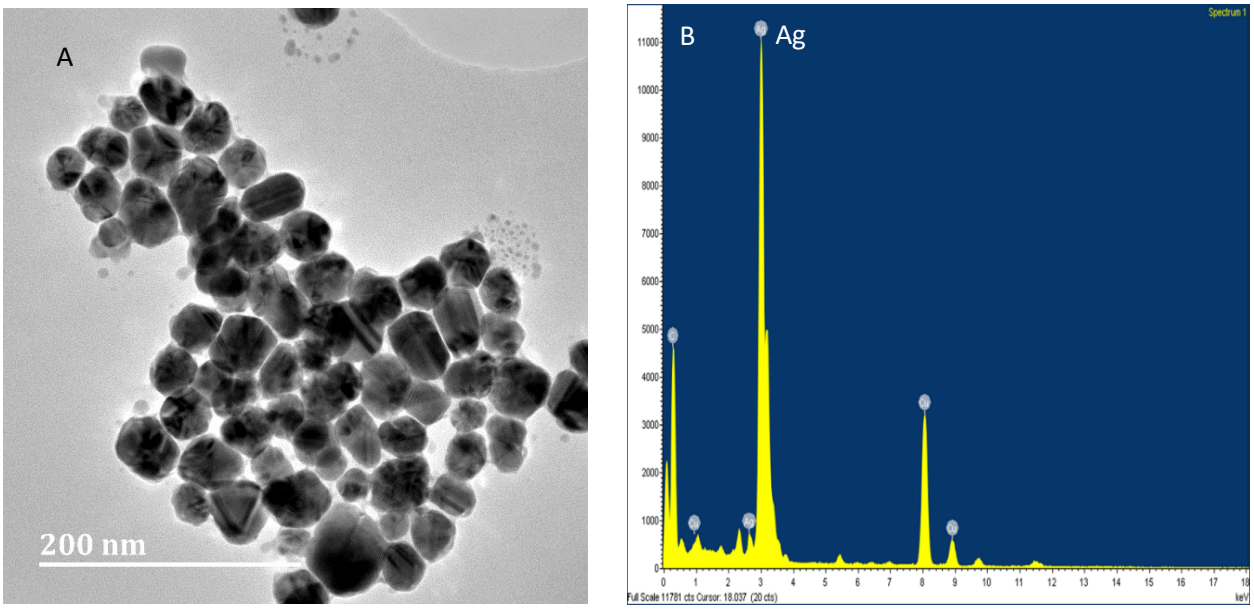


Figure 5.3: Images showing the TEM picture of silver nanoparticles (A) and EDX pattern (B).

5.4.2 TEM analysis of biologically synthesized silver nanoparticles

The synthesis of nanoparticles by microorganism was initially confirmed by the change of colour in the media (Singh, 2016). In this study the colour of modified chemically defined media with MS-1 cells changed from orange to deep brown colour. HR-TEM was used to evaluate the cell morphology of *Magnetospirillum magnetotacticum* (**Figure 5.4A**) and also revealed that silver nanoparticles are present within the cells (**Figure 5.4B**). Synthesized silver nanoparticles were poly-dispersed spherical in shape and their size ranged from 3-25 nm. The selected diffraction pattern (**Figure 5.4.C**) indicated that these silver nanoparticles are formed through reduction of metal ions. The EDX analysis confirmed that the particles were composed by elemental silver (**Figure 5.4.D**).

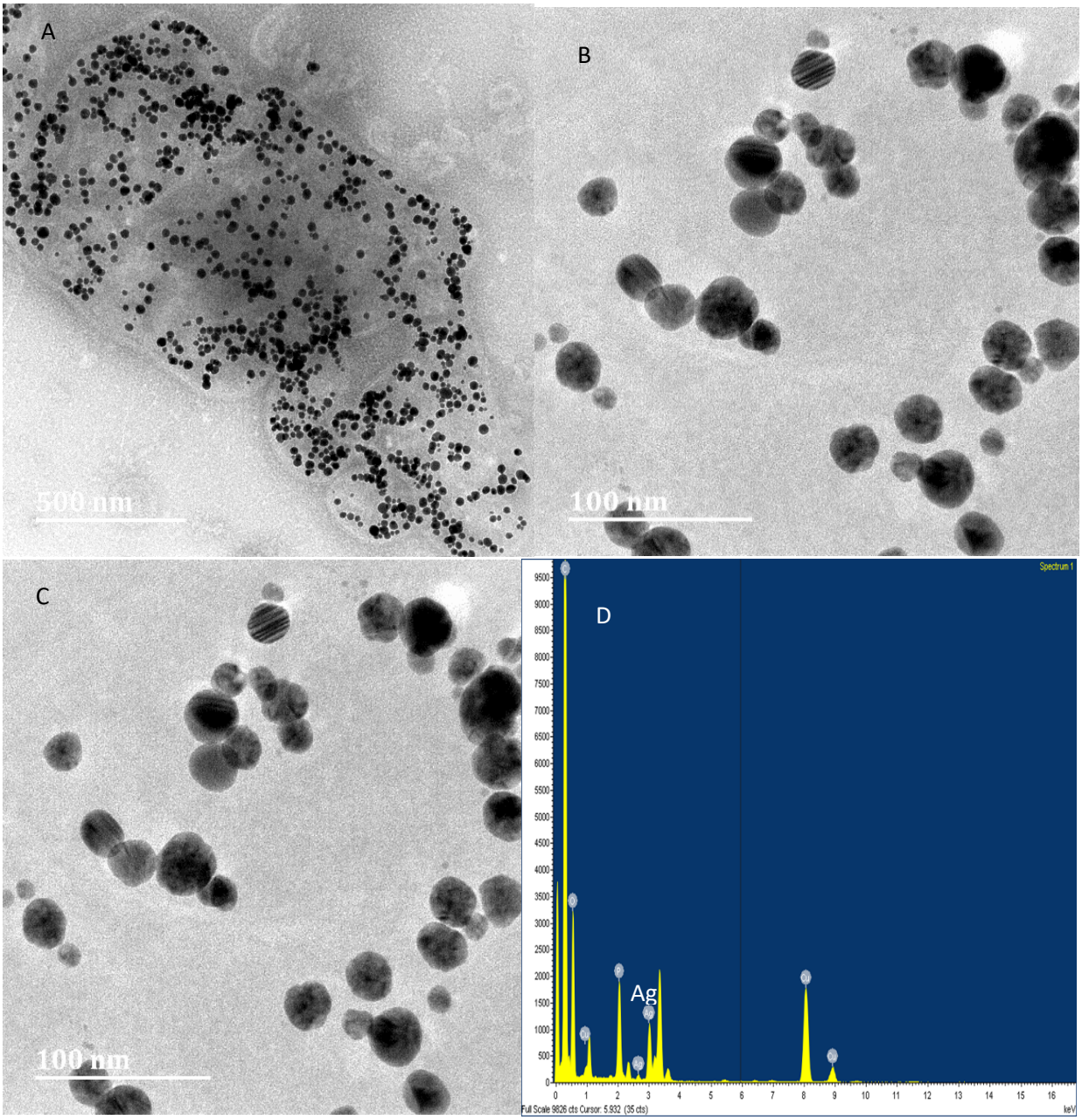


Figure 5.4: TEM images of *Magnetospirillum magnetotacticum* with silver nanoparticles (A), silver nanoparticles (B), lattice fringes (C) and EDX pattern (D).

5.4.3 Analysis of silver nanoparticles conjugated with plant extracts

About six conjugates were made from silver nanoparticles and plant extracts, three from chemically synthesized silver nanoparticles and three from biologically synthesized silver

nanoparticles. Conjugates were silver nanoparticles-methanol extract (SM), silver nanoparticles-water extract (SW), silver nanoparticles-acetone extract (SA). The measurements of FTIR were carried out in order to recognize the existence of different functional groups that results after conjugation as shown in **Figure 5.5**. Most conjugated samples compared to references sample showed the formation of -OH carbonyl and carbonyl (C=C) group, however, C-H group, (C=O) group, and C≡C group was also observed.

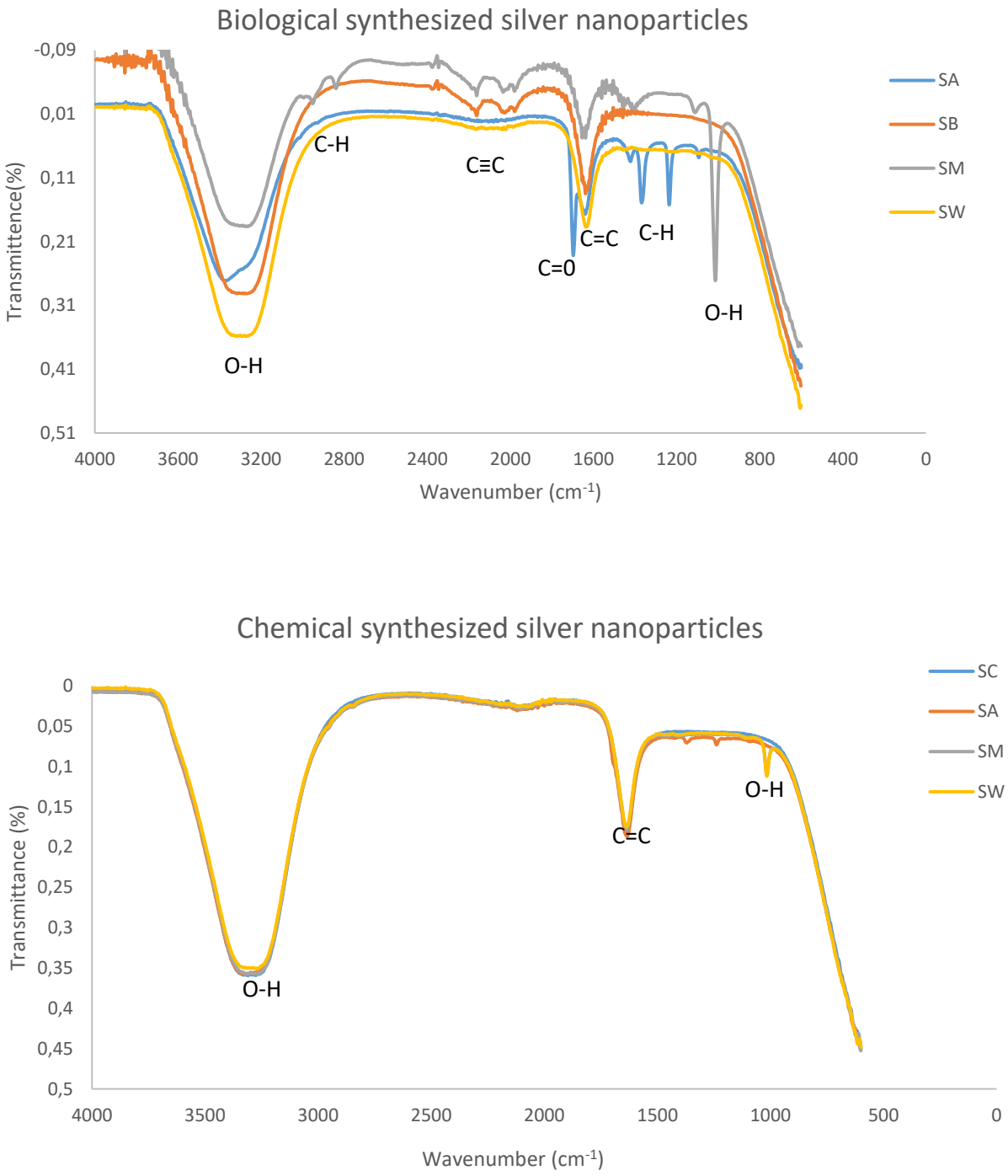
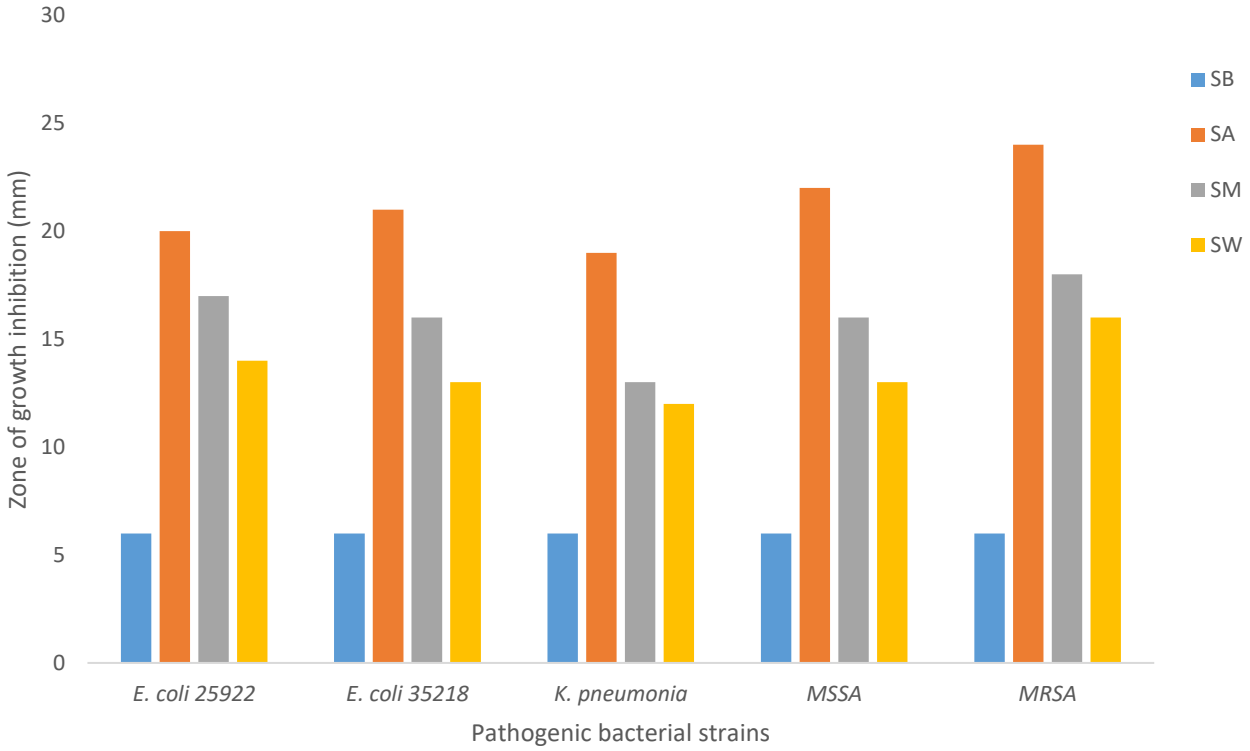


Figure 5.5: FTIR spectrum of plant extracts conjugated with silver nanoparticles where, SB (Biologically synthesized silver nanoparticles), SA (Silver nanoparticles and acetone extract), SM (Silver nanoparticles and methanol extract), SW (Silver nanoparticles and water extract) and SC (Chemically synthesized silver nanoparticles).

5.4.4 Well diffusion assay

Well diffusion assay was used to determine the antimicrobial activity of *P. grandiflora* tubers extracts when conjugated with silver nanoparticles. Biologically synthesized nanoparticles showed the smallest zones of growth inhibition of 6 mm in diameter in all bacteria tested in this study. However, higher zone of growth inhibition of 24 mm was observed with biological synthesized silver nanoparticles conjugated acetone extract against methicillin-resistant *Staphylococcus aureus* (MRSA) (**Figure 5.6**). In all bacterial strains tested with biologically synthesized silver nanoparticles, silver nanoparticles conjugated acetone extract exhibited the highest antibacterial activity with the diameter of inhibition ranging from 19-24 mm, followed by silver nanoparticles conjugated with methanol extract with a diameter ranging from 13-18 mm. Silver nanoparticles conjugated water extract showed less efficacy of antibacterial activity when compared to the other conjugates.



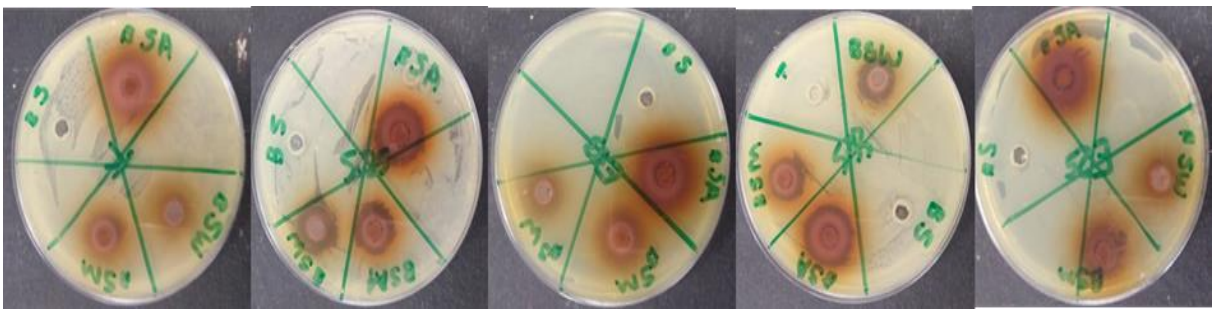


Figure 5.6: Antibacterial activity of *P. grandiflora* tuber extracts conjugated with biologically synthesized silver nanoparticles against selected bacterial strains, where SB (Biologically synthesized silver nanoparticles), SA (Silver nanoparticles and acetone extract), SM (Silver nanoparticles and methanol extract), SW (Silver nanoparticles and water extract).

Chemically synthesized silver nanoparticles exhibit good antibacterial activity against all tested bacterial strains with the diameter ranging from 21-30 mm (**Figure 5.7**). Highest antibacterial activity (31 mm) was exhibited by water extract conjugated silver nanoparticles against *E. coli* 25922 and methicillin-resistabce *Staphylococcus aureus* (MRSA) and also by acetone extract

conjugated silver nanoparticles against *S. aureus*. Water extract conjugated with silver nanoparticles also exhibited good antibacterial activity ranging from 25-27 mm.

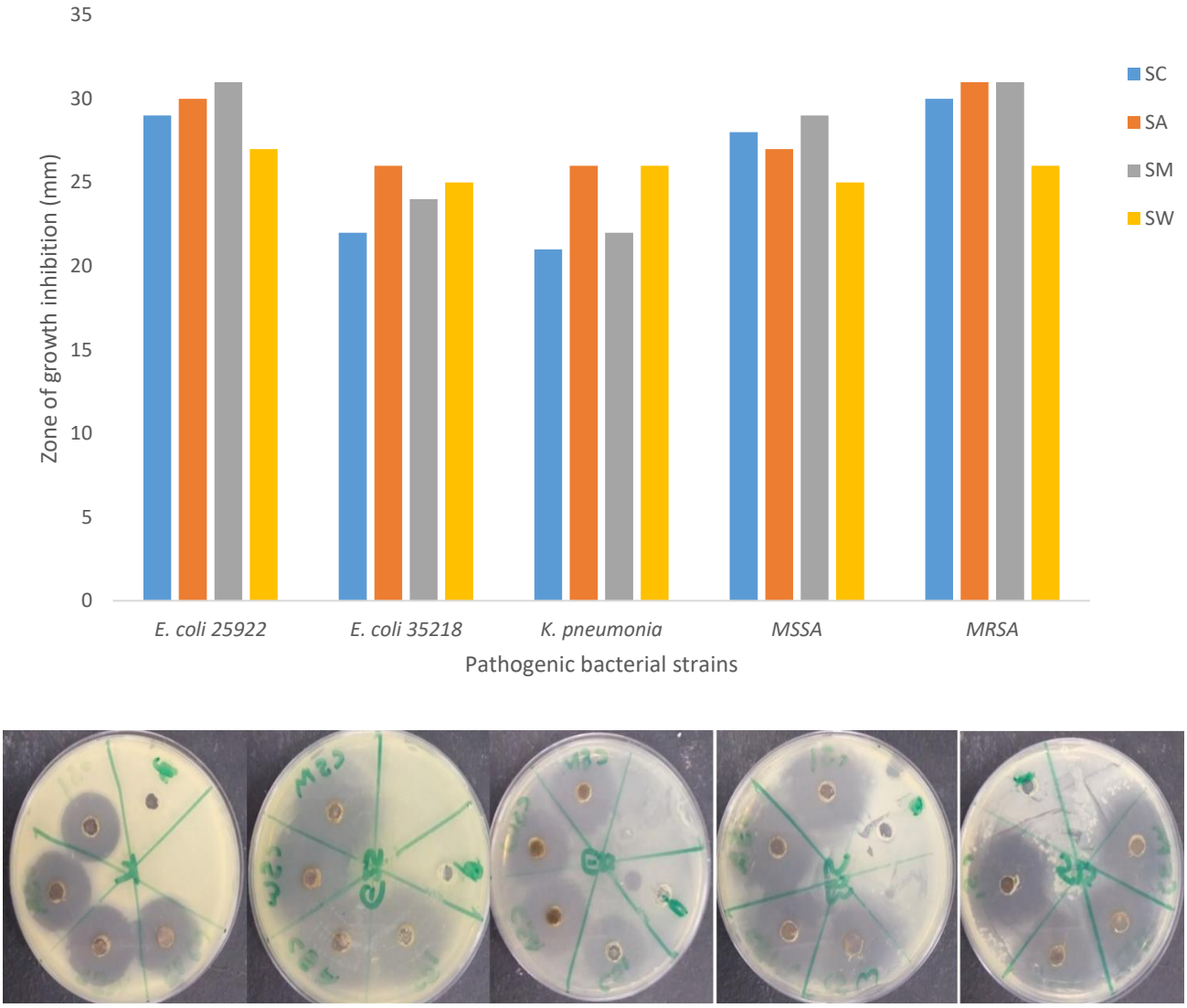


Figure 5.7: Antibacterial activity of *P. grandiflora* tuber extracts conjugated with chemically synthesized silver nanoparticles against selected bacterial strains. SC (Chemically synthesized silver nanoparticles), SA (Silver nanoparticles and acetone extract), SM (Silver nanoparticles and methanol extract), SW (Silver nanoparticles and water extract).

5.4.5 Microdilution assay

Antimicrobial activity has reported to be considered noteworthy for plant extracts when the MIC values were <1.00 mg/ ml. In this study, the concentration used ranged from 0.8 to 0.0063 mg/ml of plant extracts and their conjugates. All plant extracts showed very good antibacterial activity when conjugated with silver nanoparticles (**Table 5.1**). The lowest MIC value of 0.0063 mg/ml was observed when biologically synthesized silver nanoparticles are conjugated with acetone and water extracts against methicillin-resistant *Staphylococcus aureus* (MRSA). Chemically synthesized silver nanoparticles also shows lowest MIC value of 0.0063 mg/ml against *E. coli* 25922, and when conjugated with acetone and methanol extracts similar activity was observed against both *E. coli* and *K. pneumonia* respectively.

Table 5.1: Minimum inhibitory concentration (MIC) of biologically and chemically synthesized silver nanoparticles as well as their conjugates with *P. grandiflora* extracts.

Antibacterial agents	*E. coli* 25922	*E. coli* 35218	*K. pneumonia*	MSSA	MRSA
Biologically synthesized silver nanoparticles					
SB	0.4	0.1	0.1	0.1	0.8
SA	0.05	0.1	0.1	0.1	**0.0063**
SM	0.8	0.2	0.2	0.8	**0.0063**
SW	0.8	0.8	0.8	0.1	0.2
Chemically synthesized silver nanoparticles					
SC	**0.0063**	0.8	0.8	0.05	0.2
SA	**0.0063**	0.0125	0.0125	0.05	0.8
SM	0.4	**0.0063**	**0.0063**	0.05	0.8
SW	0.4	0.2	0.2	0.1	0.8

Key: SB (Biologically synthesized silver nanoparticles), SA (Silver nanoparticles and acetone extract), SM (Silver nanoparticles and methanol extract), SW (Silver nanoparticles and water extract) and SC (Chemically synthesized silver nanoparticles).

5.4.6 Minimum Bacterial Concentration (MBC)

Only a few conjugates were able to kill the tested bacterial strains. Methicillin-susceptible *Staphylococcus aureus* (MSSA) was killed by all conjugates of chemically synthesized silver nanoparticles (**Tables 5.2**). However, *E. coli* 25922 was killed by unconjugated silver nanoparticles and silver nanoparticles conjugated to acetone extract with MBC values of 0.4 and 0.05 mg/ml respectively, whereas *E. coli* 25922 was killed by silver nanoparticles conjugates to methanol extract with MBC value of 0.0063 mg/ml. None of the biologically synthesized silver nanoparticle conjugates were able to exhibit minimum bactericidal concentration.

Table 5.2: Minimum bactericidal concentration (MBC) of chemically synthesized silver nanoparticles as well as their conjugates.

Antibacterial agents	*E. coli* 25922	*E. coli* 35218	*K. pneumonia*	MSSA	MRSA
SC	0.4	BG	BG	BG	BG
SA	0.05	BG	BG	0.0063	BG
SM	BG	0.0063	BG	0.0063	BG
SW	BG	BG	BG	0.2	BG

Key: SC (Chemically synthesized silver nanoparticle), SA (Silver nanoparticles and acetone extract), SM (Silver nanoparticles and methanol extracts), SW (Silver nanoparticles and water extracts), and BG (Bacterial growth observed).

5.4.7 Fractional Inhibition Concentration Index (FICI) calculations

Fractional Inhibition Concentration Index was calculated for the results obtained from MIC and the values are shown in **Table 5.3**. A total of six samples from biologically and chemically

synthesized silver nanoparticles conjugated plant extracts were tested against five bacterial pathogen. A total of 7 synergies (23.3%) were observed, 9 (30%) were additive and 14 (46%) were antagonism.

Table 5.3: Effect of conjugating *P. grandiflora* tuber extracts with silver nanoparticles.

Conjugates	*E. coli* 25922	*E. coli* 35218	*K. pneumonia*	MSSA	MRSA
SA_B	**0.25 (S)**	1.125 (A)	1.125(A)	1.125(A)	**0.023625(S)**
SM_B	18 (N)	3(A)	2.5(A)	9.290323(N)	0.070875(N)
SW_B	18(N)	10(N)	134.9841(N)	16.87302(N)	**0.5(S)**
SA_C	1.01575(A)	**0.03125(S)**	**0.03125(S)**	1.0625(A)	6(N)
SM_C	71.49206(N)	**0.01575(S)**	**0.023625**	1.080645(A)	12(N)
SW_C	71.49206(N)	0.75(A)	31.99603(N)	17.87302(N)	5(N)

Key: $_B$ (Biological synthesis), $_C$ (Chemical synthesis), SA (Silver nanoparticles and acetone extract), SM (Silver nanoparticles and methanol extracts), SW (Silver nanoparticles and water extracts). A (Additive), N (Antagonism) and S (synergy).

5.5 DISCUSSION

Silver nanoparticles have been reported to have wide range of applications, is known for its antimicrobial properties and has been used for years in the medical field for antimicrobial applications and even has shown to prevent HIV binding to host cells (Sharma *et al*., 2009) In recent years, many researchers have focused on the development of modified or novel synthetic strategies for silver nanoparticles in contrast to the use of conventional methods which are

strongly associated with toxic environmental footprints (Patra and Baek, 2015; Moodley *et al.,* 2018). This study reports on the antimicrobial activities of *P. grandiflora* tuber extracts when conjugated with chemically and biologically synthesized silver nanoparticles against pathogenic bacteria.

Various microbes are known to reduce the Ag^+ ions to form silver nanoparticles, most of which are found to be spherical particles (Ahmed *et al*., 2015). In biological synthesis of silver nanoparticles, *Magnetospirillum magnetotacticum* bacteria were used and they produced spherical silver nanoparticles with the size ranging from 3-25 nm. This results are similar to the one obtained by Abhilash et al., (2011). Studies have found that many microorganisms can produce inorganic nanoparticles through intracellular or extracellular routes (Singh *et al*., 2016), in this study, TEM image of *M. Magnetotacticum* revealed that silver nanoparticles were produced intracellularly.

Is not only *Magnetospirillum* species bacteria that can produce nanoparticles, Klaus and coworkers have shown that the bacterium *Pseudomonas stutzeri* AG259 (isolated from a silver mine) when placed in a concentrated aqueous solution of silver nitrate, played a major role in the reduction of the Ag^+ ions and the formation of silver nanoparticles of well-defined size (Klaus *et al*., 1999). However, *Magnetospirillum* used in this study were mesophilic facultative anaerobic that survive at a very low concentration of oxygen (Arakaki and Matsunaga, 2003). Hence, the level of oxygen was kept at a minimum by tightly wrapping culture tubes with a parafilm. On the other hand silver nanoparticles were also chemically synthesized according to the protocol provided by Turkvich (1951) and characterization was done by UV-Vis spectroscopy and TEM.

Synthesized silver nanoparticles from biological and chemical methods were conjugated to plant extracts from *P. grandiflora* tuber. Most studies report reduction and stabilization of silver ions by combination of biomolecules such as proteins, amino acids, enzymes, polysaccharides, alkaloids,

tannins, phenolics, saponins, terpenoids and vitamins which are already established in the plant extracts having medicinal values (Singh *et al*., 2016). Moreover, plants have been reported to facilitate silver nanoparticles syntheses (Singh *et al*., 2016). Different parts of the plants which include barks, roots and leaves have been used to reduce silver nitrates into nanoparticles. With increasing intensity of extract during the period of incubation, other research study report on silver nanoparticles showed gradual change in color of the extracts to yellowish brown with callus extract of the salt marsh plant, *Sesuvium portulacastrum* (Nabikhan *et al*., 2010).

The hybrid molecules were synthesized separately and characterized by FTIR to identify functional groups that will results to confirm the conjugation process. Plant extracts when conjugated with silver nanoparticles has revealed the presence of C-H group and carbonyl (C=O) group, however, -OH carbonyl and C≡C group which were not observed in the individual solutions before conjugate. These peaks are due to the organic compounds which are present the extract and responsible for silver ions reduction and stabilization of resultant nanoparticles (Roopan *et al*., 2013). However, functionalization of biomolecules with silver nanoparticles are known to have a primary amine group, a carbonyl group, hydroxyl groups and other stabilizing functional groups as shown by FTIR spectroscopic technique (Mubarak *et al*., 2011).

In hospitals, infection is the most common complication and cause of death in patients. Therefore, antibacterial effects of silver have been incorporated into various medical applications. Plastic catheters coated with silver nanoparticles prevent biofilm formation from *E. coli*, *Enterococcus Staphylococcus aureus, Candida albicans, Staphylococci*, and *Pseudomonas aeruginosa* and also show significant *in vitro* antimicrobial activity (Sharma *et al*., 2009). In our study, well diffusion assay results clearly indicated that chemically synthesized silver nanoparticles when conjugated with *P. grandiflora* tuber extracts have good anti-bacterial activity against *E. coli*, *K. pneumonia* and *S. aureus*. Similarly, In a recent report, these nanoparticles have been synthesized on irradiation using an aqueous mixture of *Ficus carica* leaf extract (Ahmed and Ikram, 2015).

Cymbopogan citratus (DC) stapf (commonly known as lemon grass) a native aromatic herb from India and also cultivated in other tropical and subtropical countries showed strong antibacterial effect against *P. aeruginosa*, *P. mirabilis*, *E. coli*, *Shigella flexaneri*, *S. Somenei* and *K. pneumonia* (Masurkar *et al*., 2011).

The green rapid syntheses of spherically shaped silver nanoparticles with dimensions of 50–100 nm were observed using *Alternanthera dentate* aqueous extract (Kumar *et al*., 2014) when we compare this to silver nanoparticles synthesized in this study. Ours were much smaller in size than those reported by Kumar and colleagues. The silver nanoparticles reported by Kurmar exhibit antibacterial activity against *P. aeruginosa*, *E. coli*, *K. pneumonia* and *E. faecal* (Kumar *et al*., 2014). In this scenario, highest zone of growth inhibition of 24 mm was observed with biologically synthesized silver nanoparticles conjugated with acetone extract against MRSA. Nevertheless, chemically synthesized silver nanoparticles showed the highest zone of growth inhibition with diameter of 31 mm against *S. aureus* when conjugated with both acetone and methanol extracts. Hence, acetone extracts can be considered as a best extracts for conjugation since it reveal more antibacterial activity.

The MIC results revealed that all extracts were active at a very low concentration, lower than 1 mg/ml of plants extracts (Vatsos and Rebours, 2015). The study conducted by Nabikhan et al., (2010) has revealed that silver nanoparticles conjugated with *Sesuvium portulacastrum* active compounds as stabilizers showed high antimicrobial activities against *S. typhi*, *E. coli, S. aureus* and *B. subtilis* microorganisms. In this study lowest MIC was revealed when biologically synthesized silver nanoparticles are conjugated with acetone and water extract against methicillin-resistant *Staphylococcus aureus* with the MIC value of 0.0063 mg/ml. The overall synergistic effect was (23.3%), whereas (30%) were additive and (46%) were antagonism. However, most synergistic effect was observed with *P. grandiflora* acetone and water extract when conjugated with chemically synthesized nanoparticles against *E. coli* and *K. pneumoniae*.

5.6 CONCLUSION

Synthesis and characterization of silver nanoparticles was successfully carried out in this study. The *in-vitro* antibacterial activity of silver nanoparticles conjugated with *P. gandiflora* tuber extracts showed potential antibacterial property against multi-drug resistant pathogens such as *Staphylococcus aureus*, *Klebsiella pneumonia* and *Escherichia coli*. Therefore, biosynthesized conjugate could be utilized as antimicrobial agents for effective disease management due to the synergistic antibacterial activity that was observed.

5.7 *REFERENCES*

Ahmed, S. and Ikram, S., (2015). Silver nanoparticles: one pot green synthesis using *Terminalia arjuna* extract for biological application. *Journal of Nanomedical Nanotechnology*, 6(4), p.309.

Arakaki, A., Webb, J. and Matsunaga, T., (2003). A novel protein tightly bound to bacterial magnetic particles in *Magnetospirillum magneticum* strain AMB-1. *Journal of Biological Chemistry*, 278(10), pp.8745-8750.

Barabadi, H., Honary, S., Mohammadi, M.A., Ahmadpour, E., Rahimi, M.T., Alizadeh, A., Naghibi, F. and Saravanan, M., (2017). Green chemical synthesis of gold nanoparticles by using *Penicillium aculeatum* and their scolicidal activity against hydatid cyst protoscolices of *Echinococcus granulosus*. *Environmental Science and Pollution Research*, 24(6), pp.5800-5810.

Klaus, T., Joerger, R., Olsson, E. and Granqvist, C.G., (1999). Silver-based crystalline nanoparticles, microbially fabricated. *Proceedings of the National Academy of Sciences*, 96(24), pp.13611-13614.

Kumar, D.A., Palanichamy, V. and Roopan, S.M., (2014). Green synthesis of silver nanoparticles using Alternanthera dentata leaf extract at room temperature and their antimicrobial activity. Spectrochimica Acta Part A: *Molecular and Biomolecular Spectroscopy*, 127, pp.168-171.

Masurkar, S.A., Chaudhari, P.R., Shidore, V.B. and Kamble, S.P., (2011). Rapid biosynthesis of silver nanoparticles using Cymbopogan citratus (lemongrass) and its antimicrobial activity. Nano-micro letters, 3(3), pp.189-194.

Moodley, J.S., Krishna, S.B., Pillay, K. and Govender, P., (2018). Production, characterization and antimicrobial activity of silver nanoparticles produced by *Pediococcus acidilactici*. digest *journal of nanomaterials and biostructures*, 13(1), pp.77-86.

Mustafa, G., Rawaba A., Asia A., Sumaira, S. and Amer J., (2017), Bioactive Compounds from Medicinal Plants and Their Importance in Drug Discovery in Pakistan. *Matrix Science Pharma*ceutical (MSP) 1) 17-26. 2455-0986

Nabikhan, A., Kandasamy, K., Raj, A. and Alikunhi, N.M., (2010). Synthesis of antimicrobial silver nanoparticles by callus and leaf extracts from saltmarsh plant, *Sesuvium portulacastrum L*. *Colloids and Surfaces B: Biointerfaces,* 79(2), pp.488-493.

Patra, J.K. and Baek, K.H., (2015). Novel green synthesis of gold nanoparticles using *Citrullus lanatus* rind and investigation of proteasome inhibitory activity, antibacterial, and antioxidant potential. *International journal of nanomedicine*, 10, p.7253.

Ramalivhana, N.J., (2010). Molecular characterization of *Aeromonas hydrophila* and antimicrobial activities of selected medicinal plants against pathogenic isolates from water and stool samples in the era of HIV/AIDS in Limpopo Province, South Africa (Doctoral dissertation).

Ramkumar, V.S., Pugazhendhi, A., Gopalakrishnan, K., Sivagurunathan, P., Saratale, G.D., Dung, T.N.B. and Kannapiran, E., (2017). Biofabrication and characterization of silver nanoparticles using aqueous extract of seaweed *Enteromorpha compressa* and its biomedical properties. *Biotechnology reports*, 14, pp.1-7.

Rasool Hassan Bassam Abdul, (2012).Medicinal plants (Importants and uses) *Pharmaceutical Analyst Acta* 3: e139. doi:10.4172/2153-2435.1000e139

Samie, A., Obi, C.L., Bessong, P.O. and Namrita, L., (2005). Activity profiles of fourteen selected medicinal plants from Rural Venda communities in South Africa against fifteen clinical bacterial species. *African journal of Biotechnology*, 4(12).

Saratale, G.D., Saratale, R.G., Benelli, G., Kumar, G., Pugazhendhi, A., Kim, D.S. and Shin, H.S., (2017). Anti-diabetic potential of silver nanoparticles synthesized with *Argyreia nervosa* leaf

extract high synergistic antibacterial activity with standard antibiotics against foodborne bacteria. *Journal of Cluster Science*, 28(3), pp.1709-1727.

Singh, P., Kim, Y.J., Zhang, D. and Yang, D.C., (2016). Biological synthesis of nanoparticles from plants and microorganisms. *Trends in biotechnology*, 34(7), pp.588-599

Subbaiya, R., Saravanan, M., Priya, A.R., Shankar, K.R., Selvam, M., Ovais, M., Balajee, R. and Barabadi, H., (2017). Biomimetic synthesis of silver nanoparticles from *Streptomyces atrovirens* and their potential anticancer activity against human breast cancer cells. *IET nanobiotechnology*, 11(8), pp.965-972.

Tejamaya, M., Römer, I., Merrifield, R.C. and Lead, J.R., (2012). Stability of citrate, PVP, and PEG coated silver nanoparticles in ecotoxicology media. *Environmental science & technology*, 46(13), pp.7011-7017.

Tillotson, G.S., and Theriault, N., (2013). New and alternative approaches to tackling antibiotic resistance. *F1000 Prime Reports*. 5:51. doi: 10.12703/P5-51

Turkevich, J., Stevenson, P.C. and Hillier, J. (1951). A study of the nucleation and growth processes in the synthesis of colloidal gold. *Discussions of the Faraday Society*, 1951. 11: p. 55-75.

Wang, H., Chen, Y., Li, X.Y. and Liu, Y., (2007). Synthesis of oligo (ethylenediamino)-β-cyclodextrin modified gold nanoparticle as a DNA concentrator. *Molecular pharmaceutics*, 4(2), pp.189-198.

Yazdi, M.E.T., Khara, J., Sadeghnia, H.R., Bahabadi, S.E. and Darroudi, M., (2018). Biosynthesis, characterization, and antibacterial activity of silver nanoparticles using *Rheum turkestanicum* shoots extract. *Research on Chemical Intermediates*, 44(2), pp.1325-1334.

CHAPTER SIX: SYNTHESES, CHARACTERIZATION AND EVALUATION OF ANTIBACTERIAL ACTIVITY OF *P. GRANDIFLORA* CONJUGATED WITH GOLD NANOPARTICLES

6.1 SUMMARY

BACKGROUND: Non-covalent interaction of nanoparticles with biomolecules has been frequently applied in the generation of hybrid molecules. Gold nanoparticles are still attracting the attention of researchers as they possess characteristics which connect bulk materials and atomic/ molecular structure. The increase of drug resistance among pathogenic bacteria have made the search for new antimicrobials more necessary. The most promising and novel antimicrobial agents are medicinal plants and metal nanoparticles in the current situation.

OBJECTIVES: The aim of this study was to synthesize, characterize and evaluate *Pyrenacantha grandiflora* Baill extracts and gold nanoparticles conjugates against pathogenic bacteria.

METHODS: Gold nanoparticles were synthesized using chemical and biological methods. Nanoparticles were analyzed with a UV- Vis spectrophotometer, Transmission electron microscopy (TEM) and Energy Dispersive X-Ray Analysis (EDX). Gold nanoparticles were conjugated to plant extracts and analyzed with a Fourier transform infrared spectroscope (FTIR). Well diffusion assay was used to evaluate the antimicrobial activities of the conjugates. Minimum inhibitory concentrations (MIC) were used to identify the minimum concentrations needed to inhibit the growth of bacteria. Minimum bactericidal concentration were also used to assesse viability of bacteria by sub-culturing MIC result in a fresh growth media.

RESULTS: The UV–Visible spectra of gold nanoparticles showed synthesis peak at 530 nm. FTIR analysis indicated functional bio-molecules associated with plant extracts conjugated gold nanoparticles such as formation of C-H group and carbonyl (C=O) group, however, -OH carbonyl

and C≡C group was also observed. TEM revealed the star shape of biologically synthesized nanoparticles with a mean particle size of 11 nm. Well diffusion assay showed highest zone of growth inhibition of 22 mm which was observed in biologically synthesized gold nanoparticles conjugated water extract against methicillin-susceptible *Staphylococcus aureus*. Chemically synthesized gold nanoparticles and its conjugates exhibited good antibacterial activity against all tested bacterial strains. The MIC revealed the lowest value of 0.0063 mg/ml which was observed when biologically synthesized gold nanoparticle are conjugated with acetone and water extracts against *K. pneumonia*. From the MIC results, the activity of acetone extracts is improved with chemically synthesized gold nanoparticles against *E. coli* and methicillin-resistant *Staphylococcus aureus*. Synergistic effect was observed against all tested bacteria, except MRSA when gold nanoparticles are conjugated to acetone extract.

CONCLUSION: Overall, we reported the synthesis and characterization of *P. grandiflora* tuber extracts conjugated with gold nanoparticles. Hence, they showed a very good antibacterial activity which improved both plant extracts and gold nanoparticles individual activity.

Keywords: Antibacterial activity, Gold nanoparticles, Plant extracts, Agar diffusion assay, Minimum bactericidal concentration.

6.2 INTRODUCTION

New strategies are being developed to improve existing medicine to combat pathogenic resistant of microorganisms. Recently, the use of the inorganic material in conjugation with organic drug molecules in combination therapy has drawn much attention (Pelgrift, 2013). Plants extracts and microorganisms seem to be the best candidates and they are suitable for large-scale biosynthesis of nanoparticles (Iravani, 2011). Nanoparticles produced by plants are more stable and the rate of synthesis is faster than in the case of microorganisms. Moreover, the nanoparticles produced from chemical synthesis are more varied in shape and size in comparison with those produced by other organisms (Sun and Xia, 2002).

The advantages of using plant and plant-derived materials for biosynthesis of metal nanoparticles have interested researchers to investigate mechanisms of metal ions uptake and bioreduction by plants, to understand the possible mechanism of metal nanoparticle formation in plants (Iravani, 2011). Nanoparticles of noble metals, such as gold, silver, and platinum, are widely applied in products that directly come in contact with the human body, such as shampoos, soaps, detergent, shoes, cosmetic products, and toothpaste, besides medical and pharmaceutical applications. Gold has a long history of use (Bhattacharya, 2012).

Red colloidal gold has been used as medicine for revitalization in China and India Gold nanoparticles have found use in diagnostic and drug delivery applications (Bhumkar *et al*., 2007). Therefore, there is a growing need to develop environmentally friendly processes for nanoparticle synthesis without using toxic chemicals. In this approach, drugs can be functionalized on the solid core surface of metal nanoparticles either by covalent or non-covalent interactions (Agostoni *et al.*, 2015). Covalent linking requires additional chemical modification of drug molecules, which could bring about modification of the properties of drug molecules (Gannimani *et al*., 2016). Nevertheless, non-covalent strategies to functionalize the drug molecules on the surface of

nanoparticles involve only electrostatic and van der Waal's interactions, and accordingly drugs can be utilized in its native form (Gannimani *et al*., 2016). This study aimed to evaluate antibacterial the activity of *Pyrenacantha grandiflora* Baill extracts when conjugated with gold nanoparticles against selected pathogenic bacteria (*Staphyloccocus aureus* (MRSA), *Escherichia coli*, *Klebsiella pneumoniae*).

6.3 MATERIALS AND METHODS

6.3.1 Chemicals and reagents used

Chloroauric acid (HAuCl4) was purchased from Sigma (USA). Tri-sodium citrate was purchased from Rochelle chemicals (SA). All chemicals were molecular grade.

6.3.2 Microorganisms and growth media

The microorganisms that were used in this study include *Magnetospirillum magnetotacticum* MS-1 (American Type Culture Collection) which was grown in modified chemically defined growth medium supplemented with ISOGRO. Methicillin-resistant *Staphylococcus aureus* ATCC 25923, methicillin-susceptible *Staphylococcus aureus* ATCC 33594 were sub-cultured on mannitol salt agar (Neogen, Michigan). *Escherichia coli* ATCC 35218 and 25922 were sub-cultured on MacConkey agar (Oxoid, England) and *Klebsiella pneumonia* ATCC 700603 was sub-cultured on nutrient agar (Rochelle, SA). An inoculum of each bacterial strain was suspended in 5 ml of Mueller Hinton broth (Rochelle, SA) and incubated for 3 hours at 37^{o}C. The cultures were diluted with Mueller Hinton Broth and adjusted to give a concentration of bacterial cells equivalent to a 0.5 McFarland standard prior to the antibacterial testing.

6.3.3 Biological synthesis of gold nanoparticles using bacteria

6.3.3.1 Cultivation of *Magnetospirillum magnetotacticum* bacteria

Gold nanoparticles was synthesized using *Magnetospirillum magnetotacticum* MS-1. These bacteria were cultured in chemically defined growth media prepared with slight modification. ISOGRO was added in the media to enhance the growth of bacteria by shortening the lag phase of growth resulting in high bacterial yield. Tetrachloroauric acid (Sigma, USA) which was added as a source of metal in the growth media. The MS-1 cells were grown for 4 days at 30°C in airtight 50 ml Falcon tubes wrapped with parafilm because they grow well in micro-aerobic condition. Tubes were covered with foil with foil to prevent photo-degradation of gold chloride in the media. Synthesis of gold nanoparticles was initially confirmed by a color change in the media from orange to ruby red color.

6.3.3.2 Analysis of gold nanoparticles synthesis by TEM

High- Resolution Transmission Electron Microscopy (HR-TEM) was used to detect the whether gold nanoparticles produced by *Magnetospirillum magnetotacticum* cells. The samples were prepared by placing a drop of the bacterial culture onto a carbon-coated copper TEM grid. The sample was then dried under an infrared lamp for a period of 1 hour. High-resolution TEM images were obtained on JEOL TEM model no 2100 instrument operated at an accelerating voltage of 200 kV and 0.23 nm resolution.

6.3.3.3 Isolation of gold nanoparticles

The isolation of gold nanoparticles from *Magnetospirillum magnetotacticum* culture was done using MACS magnetic separation column (Miltenyi Biotec). Briefly, *M. magnetotacticum* bacterial cells were suspended in 20 mM HEPES-4 mM EDTA (pH 7.4) and broke open by sonication for 5 min at 21°C. The unbroken cells were removed by centrifugation at 9000 rpm for 30 min. The supernatant was harvested and passed through MACS magnetic separation column following manufacturer's protocol. Unbound magnetic particles were washed using 10 mM HEPES-200 mM NaCl (pH 7.4) then silver nanoparticles were eluted with 10 mM HEPES (pH 7.4). UV-VIS spectrophotometer was used to analyze the formation of gold nanoparticles at 500-600 nm.

6.3.4. Gold nanoparticles preparation

The gold nanoparticles were prepared according to the method described previously (Leopold *et al*., 2013). Polyethylene glycol (PEG)-400 was used as reducing agent for the tetrachloroauric acid. To obtain the PEG-AuNPs, 600 µl of PEG400 was dissolved in 95 ml of water; then 900 µl of 1% sodium hydroxide was added to the PEG solution for pH adjustment (pH around 8). Afterward, 600 µl of 2% $HAuCl_4$ solution were rapidly added to the boiling PEG solution under stirring. The mixture was further boiled for five minutes. A wine-red colloid was rapidly formed which confirmed formation of gold nanoparticles.

6.3.4.1 UV-Vis Spectrum analysis

Characterization of gold nanoparticles was done using UV-Visible Spectrophotometer (Specord 210, Analytikjena spectrometer). The reduction of chloroauric acid to gold nanoparticles by PEG was confirmed by observing a broad absorbance peak between 500-600 nm.

6.3.4.1 High Resolution- Transmission Electron Microscopy

Further characterization was done using HR-TEM studies. The sample was prepared by placing a drop of the nanoparticle solution onto a carbon-coated copper TEM grid. The sample was then dried under an infrared lamp for a period of 45 min for solvent to evaporate. High-resolution TEM images were obtained on JEOL TEM model no 2100 instrument operated at an accelerating voltage of 200 kV and 0.23 nm resolution.

6.3.5 Preparation of plant extract conjugated with gold nanoparticle

Pyenacantha grandiflora extracts were prepared using acetone, methanol, and water as a solvent as explained in Chapter 3. Non-covalent modification of plants extract with gold nanoparticles was applied in the generation of hybrid molecules. Synthesized gold nanoparticles from both chemical and biological methods were mixed with 2 ml of 10 mg/ml of each plant extract (acetone, water, and methanol extracts) and incubated at 4ºC for 24 hours.

6.3.6 Characterization of conjugates

All conjugates were analyzed using Fourier- transmission electron microscopy (FTIR) in a range of 400-4000 cm^{-1} to detect various functional groups formed after conjugation that may be responsible for biological activities. About 500 µl of dried conjugates were placed on the sample chamber of FTIR spectrophotometer and the spectra were recorded in the scan range of 400–4000 cm^{-1} with a resolution of 4 cm^{-1} on Nicolet Avatar 330 FTIR spectrometer.

6.3.7 Well diffusion assay

The antibacterial activities of the conjugates were initially determined by well diffusion method. The zones of inhibition were recorded in millimeter (mm). Briefly, bacterial suspensions were prepared with the turbidity of 0.5 McFarland. Mueller-Hinton agar plates were inoculated with *E. coli, K. pneumonia* and *S. aureus*. Wells with a diameter of 6 mm were cut using a cork borer and filled with 30 µl of the conjugates and reference samples (plant extracts and silver nanoparticles). Distilled water was used as a negative control. Plates were incubated for 24 hours at 37°C. After incubation, the growth inhibition zone diameters were measured.

6.3.8 Microdilution assay

The minimum dilution of all extracts plus conjugates samples that inhibits the growth of the microorganism were denoted as minimum inhibitory concentration (MIC) (Samie et al., 2005). Distilled water was used as negative control and gentamycin was used as positive control. After adding INT (iodo-nitro tetrazolium), the results were read by observing the color change and determining the MIC. All the extracts and AuNPs that showed activity (reduced or no color change)

were inoculated again in the agar plate and incubated overnight to determine the minimum bactericidal concentration (MBC).

6.3.9 Fractional Inhibition Concentration Index (FICI) calculations

Determination of the mutual influence of *P. grandiflora* tubers extracts and silver nanoparticles in conjugate was done using Fractional Inhibition Concentration Index by the following formula:

$$FICI = \frac{MIC\ of\ AB}{MIC\ of\ A} + \frac{MIC\ of\ AB}{MIC\ of\ B}$$

Where AB represent a combination of *P. grandiflora* tubers extracts (A) and gold nanoparticles (B). Results were interpreted as synergy (FICI≤0.5), antagonism (FICI>4) and no interaction or additive (FICI>0.5-4.0).

6.4 RESULTS

6.4.1 TEM analysis of biologically synthesized of gold nanoparticles

The color of modified chemically defined media with MS-1 bacteria change from orange to ruby red colour indicating the successful formation of gold nanoparticles. HR-TEM was used to evaluate the cell morphology of *Magnetospirillum magnetotacticum* (**Figure 6.1A**) and also reveal that gold nanoparticles were present within the cells (**Figure 6.1B**). Synthesized gold nanoparticles were star-shaped with their sizes ranging from 9-30 nm. The selected diffraction pattern (**Figure 6.1.C**) indicate that these gold nanoparticles were formed from reduction of metal

ions. The EDX analysis confirmed that the particles were composed of elemental gold (**Figure 6.1.D**).

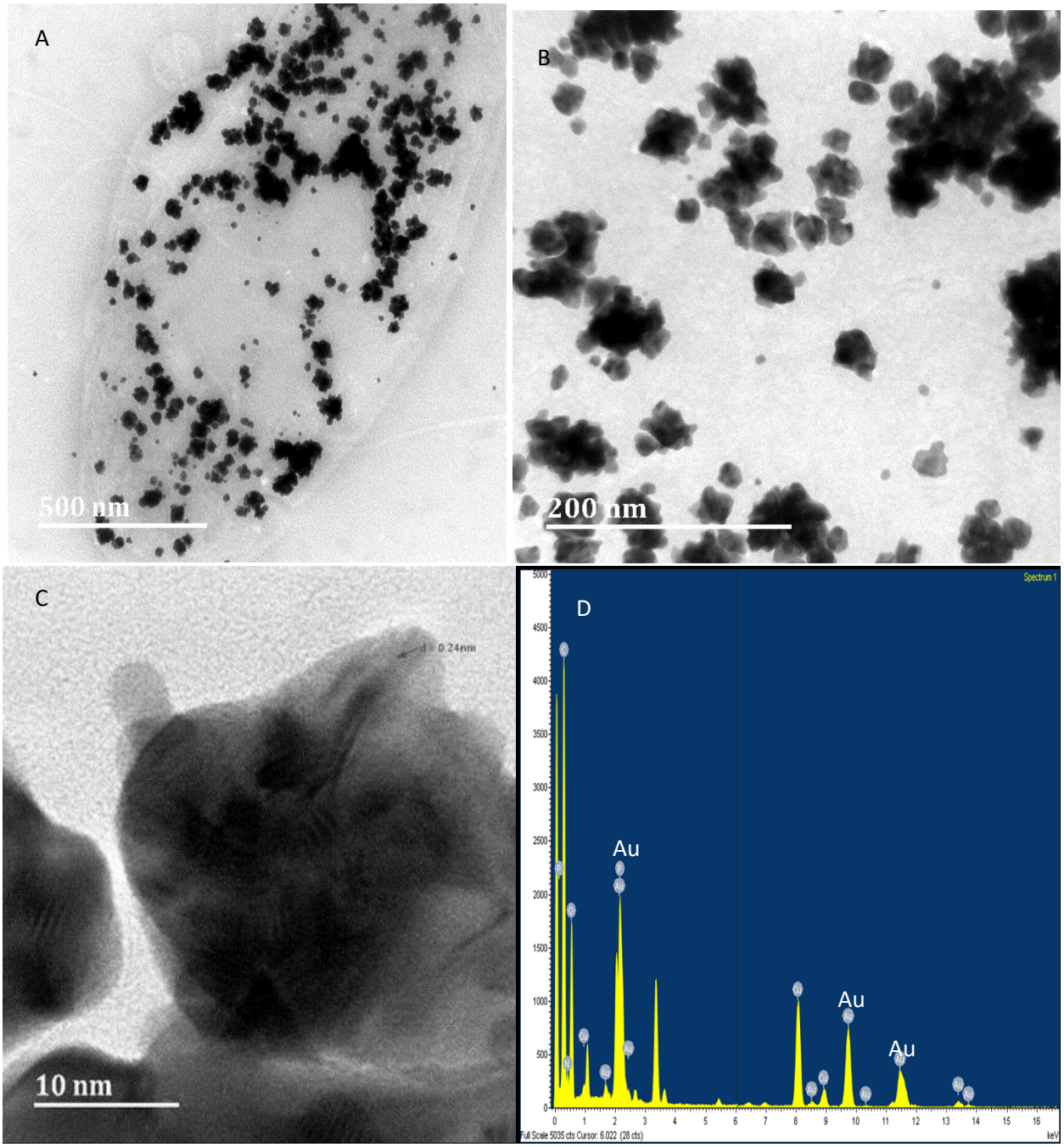


Figure 6.1: TEM images of *Magnetospirillum magnetotacticum* with gold nanoparticles (A), gold nanoparticles (B), lattice fringes (C) and EDX pattern (D).

6.4.2 Analysis of PEG capped gold nanoparticles

Gold nanoparticles capped with PEG were successfully synthesized using the chemical method. Synthesis of gold nanoparticles was initially confirmed by a color change in PEG-gold chloride solution from pale yellow (**Figure 6.2A**) to ruby red color (**Figure 6.2B**) giving rise to a characteristic absorption of light at a wavelength of 500-600 nm.

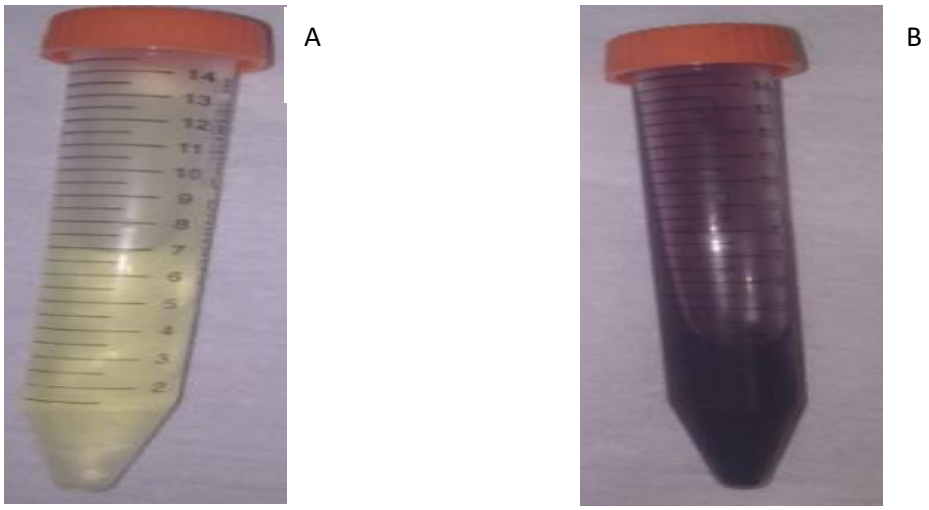


Figure 6.2: Images illustrating the color change of PEG-gold chloride solution from pale yellow (A) to ruby red (B) to confirm the synthesis of gold nanoparticles.

6.4.2.1 UV-Visible analysis

UV-Visible Spectroscopic studies of chemically synthesized gold nanoparticles showed the peak at 530 nm (maximum absorbance) which confirms the presence of gold nanoparticles as shown in **Figure 6.3**.

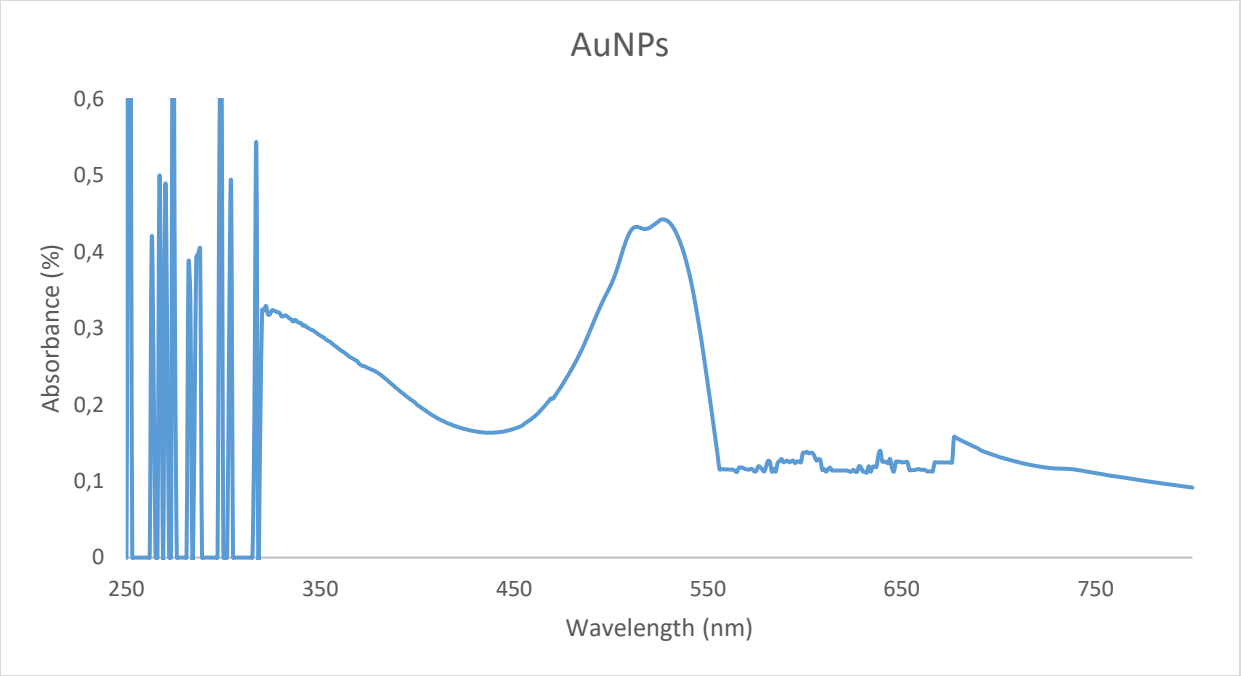


Figure 6.3: UV–Visible spectra of chemically synthesized gold nanoparticles.

6.4.2.2 TEM analysis

TEM revealed that the morphology of nanoparticles is spherical in shape. In the higher magnification image, gold nanoparticles of 7-16 nm diameter were also observed. However, the average size of the nanoparticles was found to be 11 nm. **Figure 6.4 (A)** shows the TEM picture of gold nanoparticles. The elemental composition was confirmed by EDX (**Figure 6.4 B).**

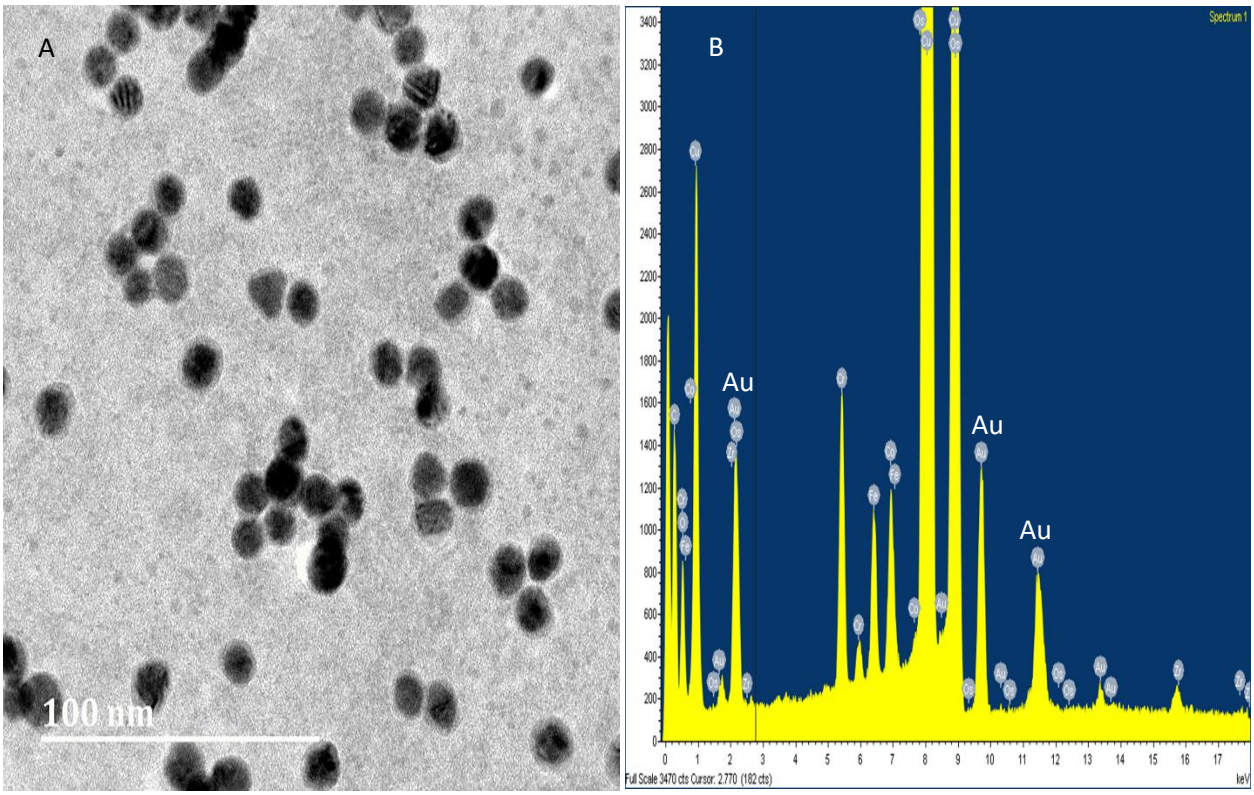
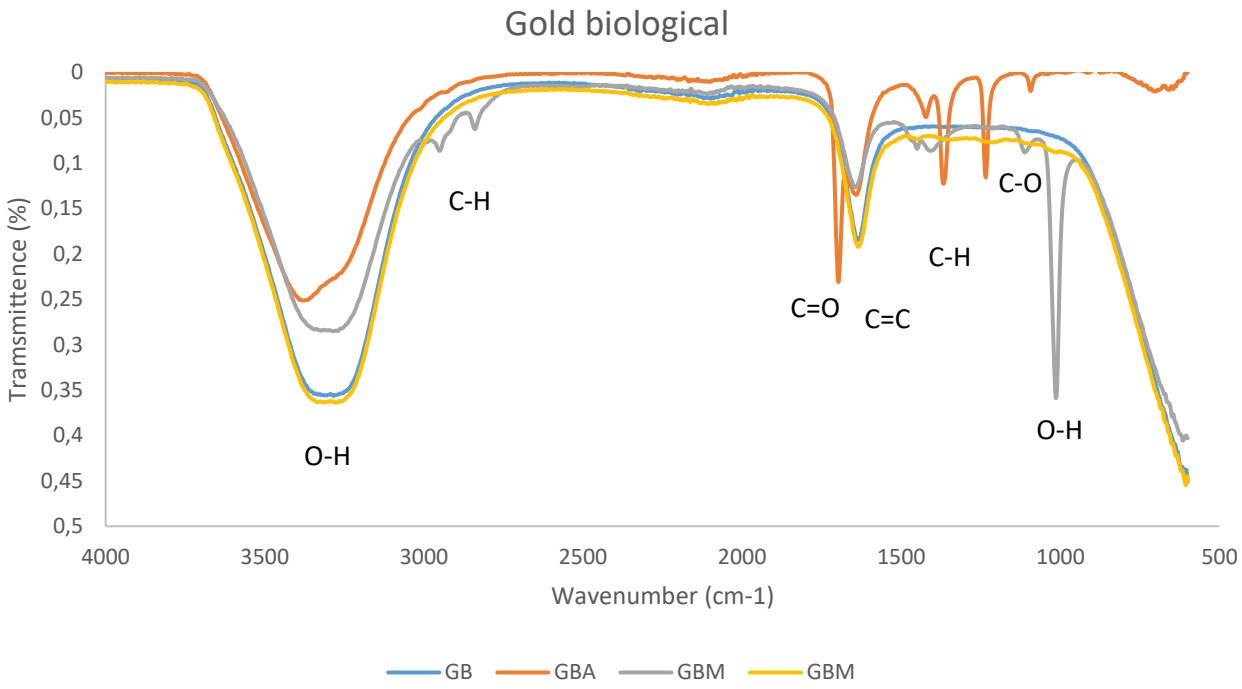


Figure 6.4: TEM image of gold nanoparticles (A) and EDX pattern (B).

6.4.3 Analysis of gold nanoparticles conjugated with plants extract

Six conjugates were made from gold nanoparticles and plant extracts, three from chemically synthesized gold nanoparticles and three from biologically synthesized gold nanoparticles. Conjugates were gold nanoparticles-methanol extracts (GM), gold nanoparticles-water extracts (GW), and gold nanoparticles-acetone extracts (GA). The measurements of FTIR were carried out in order to recognize the existence of different functional groups that results after conjugation (**Figure 6.5**). Most conjugated samples compared to reference samples showed the formation of C-H group and carbonyl (C=O) group, however, -OH carbonyl was also observed.



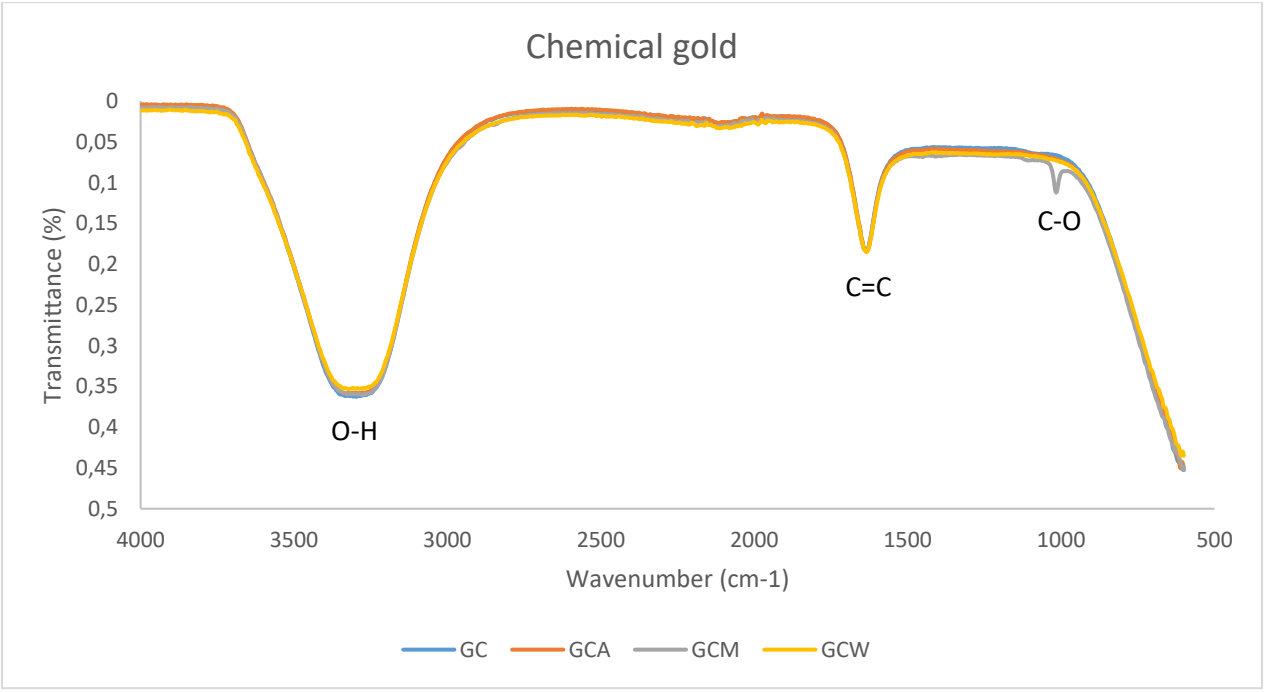


Figure 6.5: FTIR spectrum of plant extracts conjugated with gold nanoparticles.

6.4.4 Well diffusion assay

The antibacterial activity of *P. grandiflora* tuber extracts conjugated with gold nanoparticles were evaluated using well diffusion assay. Five pathogenic bacterial ATCC strain were used to evaluate the efficacy of the conjugates. Unconjugated biologically synthesized nanoparticles showed the smallest zone of growth inhibition of 6 mm in diameter in all bacteria tested in this study except in *E. coli* ATCC 35218 which was 7 mm in diameter (**Figure 6.6**). However, the highest zone of growth inhibition of 22 mm was observed when conjugated water extract against methicillin-susceptible *Staphylococcus aureus* and gold nanoparticles conjugated acetone extract against methicillin-susceptible *Staphylococcus aureus*.

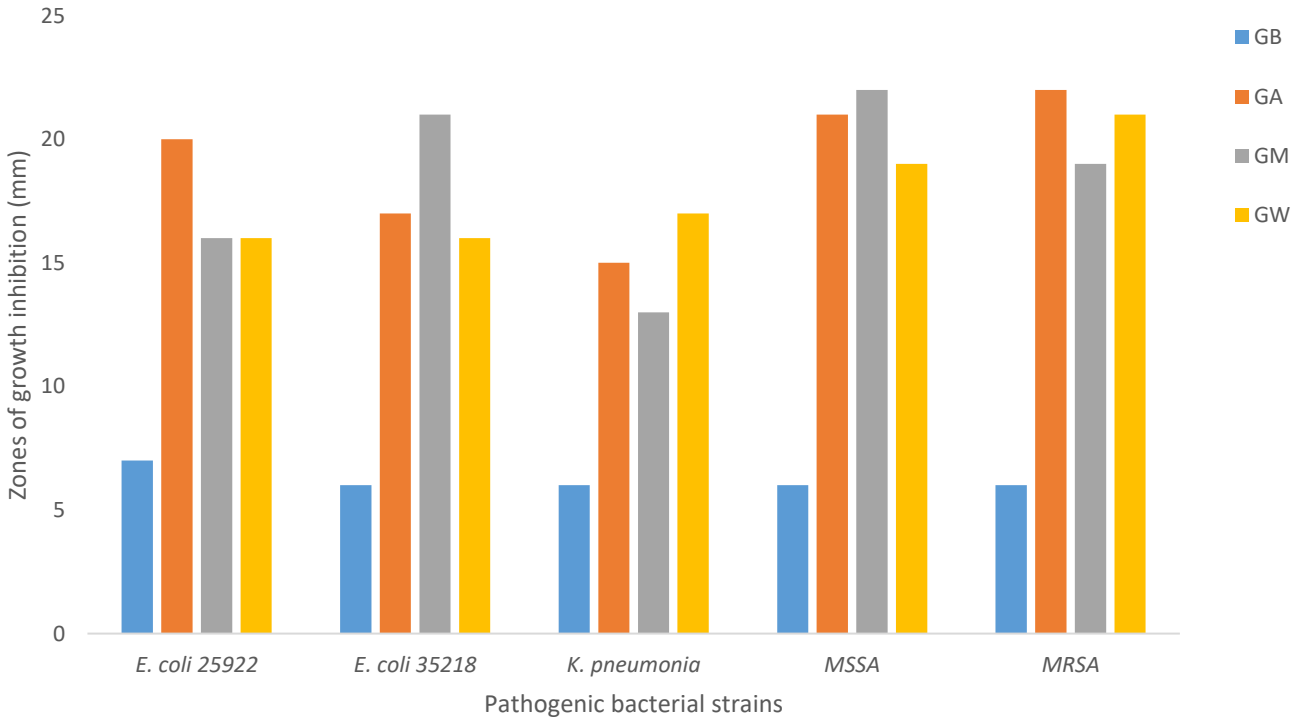


Figure 6.6: Antibacterial activity of *P. grandiflora* tubers extracts conjugates with biologically synthesized gold nanoparticles on selected pathogenic bacteria, where GB (Biologically synthesized gold nanoparticles), GA (Gold nanoparticles and acetone extract), GM (Gold nanoparticles and methanol extract), GW (Gold nanoparticles and water extract).

Chemically synthesized gold nanoparticles and its conjugates exhibited good antibacterial activity against all tested bacterial strains with the diameter ranging from 14-27 mm (**Figure 6.7**). The highest antibacterial activity (27 mm) was exhibited by gold nanoparticles alone against MSSA. *Klebsiella pneumoniae* showed the smallest zones of inhibition of all chemically synthesized gold nanoparticles and conjugates with the lowest zone of growth inhibition diameter of 14 mm when compared to other bacterial strains. However, overall good antibacterial activity was observed against *E. coli* ATCC 35218 with a range of 22-25 mm of growth inhibition zones.

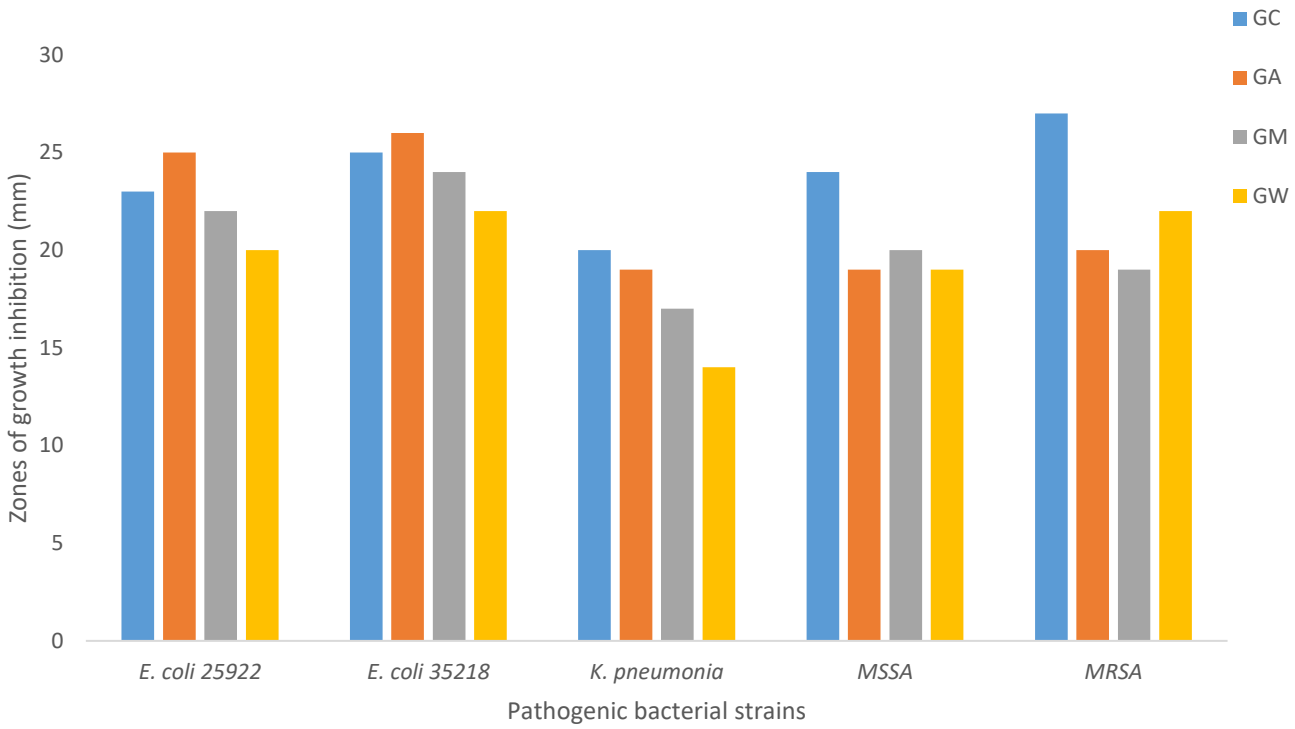


Figure 6.7: Antibacterial activity of *P. grandiflora* tubers extracts conjugates with chemically synthesized gold nanoparticles on selected pathogenic bacteria. GC (Chemically synthesized Gold nanoparticles), GA (Gold nanoparticles and acetone extract), GM (Gold nanoparticles and methanol extract), GW (Gold nanoparticles and water extract).

6.4.5 Microdilution assay

Microdilution assay was used to determine the minimum inhibitory concentration of *P. grandiflora* tubers extracts conjugated with biologically and chemically synthesized gold nanoparticles. The concentration used ranged from 0.8 to 0.0063 mg/ml of plant extract conjugated gold nanoparticles. All plant extracts showed good antibacterial activity when conjugated with gold nanoparticles (**Table 6.1**). The lowest MIC value of 0.0063 mg/ml was observed when biologically synthesized gold nanoparticles are conjugated with acetone and water extract against *K. pneumonia*. Chemically synthesized gold nanoparticles also showed lowest MIC value of 0.0063 mg/ml against *E. coli* 25922 and MSSA when conjugated with acetone extracts.

Table 6.1: Minimum inhibitory concentration (MIC) of biologically and chemically synthesized gold nanoparticles as well as their conjugates with *P. grandiflora* extracts.

Samples	*E. coli* (ATCC 25922)	*E. coli* (ATCC 35218)	*K. pneumonia* (ATCC 700603)	MSSA	MRSA
G_B	0.05	0.1	0.0125	0.05	0.8
GA_B	0.05	0.1	0.0063	0.2	0.2
GM_B	0.4	0.2	0.8	0.05	0.2
GW_B	0.4	0.8	0.0063	0.1	0.2
G_C	0.4	0.8	0.8	0.025	0.1
GA_C	0.0063	0.1	0.1	0.0063	0.8
GM_C	0.05	0.1	0.1	0.05	0.4
GW_C	0.1	0.1	0.1	0.05	0.1

Key: $_B$ (Biological synthesis), $_C$ (Chemical synthesis), G (Gold nanoparticles), GA (Gold nanoparticles with acetone extracts), GM (Gold nanoparticles with methanol extracts) and GW (Gold nanoparticles with water extracts)

6.4.6 Minimum Bactericidal Concentration (MBC)

Only unconjugated biologically synthesized gold nanoparticles and its conjugate to acetone extract were able were able to kill *E. coli* 25922 with MBC value of 0.05 and 0.8 mg/ml. None of the chemically synthesized gold nanoparticles conjugates were able to exhibit bactericidal activity.

6.4.7 Fractional Inhibition Concentration Index (FICI) calculations

Fractional Inhibition Concentration Index was calculated based on the results obtained from MIC and the results were recorded in **Table 6.2**. A total of six samples from biologically and chemically synthesized gold nanoparticles conjugated plants extracts were tested against five bacterial ATCC strains. A total of 8 synergies (26.7%) were observed, 12 (40%) were additive and 10 (33.3%) were antagonism.

Table 6.2: Effect of conjugating *P. grandiflora* tubers extracts with gold nanoparticles.

Samples	*E. coli* (ATCC 35218)	*E. coli* (ATCC 35218)	*K. pneumonia* (ATCC 35218)	MSSA	MRSA
GA_B	1.125 (A)	1.125(A)	**0.511875(S)**	4.25(N)	0.75(A)
GM_B	16(N)	3(A)	66(N)	1.080645(A)	2.25(A)
GW_B	16(N)	10(N)	1.504(A)	17.87302(N)	**0.5(S)**
GA_C	**0.0315(S)**	**0.25(S)**	**0.25(S)**	**0.259875(S)**	10(N)
GM_C	1.125(A)	0.625(A)	**0.375(S)**	2.080645(A)	8(N)
GW_C	2.25(A)	**0.375(S)**	15.99802(N)	9.936508(N)	1.125(A)

Key: $_B$ (Biological synthesis), $_C$ (Chemical synthesis), A (Additive), N (Antagonism) and S (synergy). G (Gold nanoparticles), GA (Gold nanoparticles with acetone extracts), GM (Gold nanoparticles with methanol extracts) and GW (Gold nanoparticles with water extracts).

6.5 DISCUSSION

Most of the nanoparticles are modified with functionalizing agents in order to conjugate various drugs for successful application in therapeutic purposes (Bhattacharya *et al*., 2012). In this study gold nanoparticles were conjugated with *P. grandiflora* tuber extacts and their antibacterial activity were evaluated. Gold nanoparticles were synthesized using biological and chemical methods. The biological synthesis of gold nanoparticles with *Magnetospirillum magnetotacticum* was chosen due to several advantages like simple, single step, environmentally friendly, cost effectiveness and biocompatible nature of synthesized gold nanoparticles (Singh *et al*., 2012). Additionally, there is no need to add any external stabilizing agents because biogenic components of these bacteria act as stabilizing as well as capping agents (Cai *et al*., 2011). It has been proposed that proteins, amino acids, organic acid, vitamins, as well as secondary metabolites, such as flavonoids, alkaloids, polyphenols, terpenoids, heterocyclic compounds, and polysaccharides, have significant roles in metal salt reduction and, furthermore, act as capping and stabilizing agents for synthesized nanoparticles (Singh *et al*., 2016). However, stabilizing agents are needed in chemically synthesized gold nanoparticles. Therefore, this study has used biodegradable co-polymers with hydrophilic segments which were polyethylene glycol (PEG) for surface coating of gold nanoparticles. Studies showed that PEG conformation at the nanoparticle surface is of utmost importance for the opsonin repelling function of the PEG layer (Abtahi, 2013).

Most microorganism-based synthesis for nanoparticles are slow with low productivity. To overcome such hindrances ISOGRO (a media is required for overcoming the growth limitations of minimal media) was added in the media to enhance the growth of the microorganisms and increased the yield of nanoparticles. Furthermore, problems related to microorganism-based synthesis for nanoparticles also include the complex steps, such as microbial sampling, isolation, culturing, and maintenance. Mukherjee et al., (2001) have reported the extracellular synthesis of gold nanoparticles by fungus *Fusarium oxysporum* and *Actinomycete Thermomonospora* sp.,

respectively. In this study, *Magnetospirillum magnetotacticum* has synthesized gold nanoparticles intracellularly. Several studies reported the intracellular synthesis of gold nanoparticles by *Magnetospirillum* sp. as well (Cai *et al*., 2011).

Southam and Beveridge (1996) have demonstrated that gold particles of nanoscale dimensions may readily be precipitated within bacterial cells by incubation of the cells with Au3+ ions. AuNPs of 13 nm and above are considered non-cytotoxic (Jahnen-Dechent and Simon, 2008). In this study, TEM revealed the synthesis of 7-16 nm with the chemical method and 9-30 nm nanoparticles with the biological method which further supports the size of AuNP obtained from TEM (Haiss *et al*., 2007; Amendola and Meneghetti, 2009). Nanoparticles below 2 nm have been shown to possess active cytotoxic characteristics (Schmid, 2008). Lengke et al. (2007) claimed the synthesis of gold nanostructures in different shapes (spherical, cubic, and octahedral) by filamentous cyanobacteria from Au (III)-chloride complexes. Therefore, controlled size and shape of gold nanoparticles in future will be interesting. The EDX pattern obtained is consistent with earlier reports (Kumar *et al*., 2014).

Conjugations were identified by observing new functional groups with FTIR spectroscopy. After the conjugation process the formation of C-O groups and OH hydroxyl group on conjugated plant extracts showed the presence of the new bands at 1536 and 1095 cm^{-1}. These results indicated that the conjugation between the gold nanoparticles and plant extracts actually took place. It has been stated that either through free amino groups or cysteine residues, the protein can bind to gold nanoparticles that lead to the stabilization of gold nanoparticles by surface bound protein (MubarakAli *et al*., 2011). It was reported that the ketone is the major component in *Cymbopogon flexuosus* extract that renders the liquid like characteristics of the spherical gold nanoparticle (Ahmed and Ikram, 2015).

The cell wall of Gram positive bacteria composed of a thick peptidoglycan layer, consisting of linear polysaccharide chains cross linked by short peptides, thus forming more rigid structure leading to difficult penetration of the nanoparticles, while in Gram negative bacteria the cell wall possesses thinner peptidoglycan layer (Shrivastava *et al*., 2007). Minimum inhibition concentration of *P. grandiflora* tuber extracts conjugated with biologically and chemically synthesized gold nanoparticles was determined using microdilution assay. The concentration used ranged from 0.8 to 0.0063 mg/ml, even though it has been reported that some plant extracts alone do not work (Luseba et al., 2007), all plant extracts show very good antibacterial activity when conjugated with gold nanoparticles. In 2003, Munckhof and colleagues revealed that it's not easy for unconjugated plant extracts to completely kill the bacterium. Yet, only a few chemical synthesized gold nanoparticles and their conjugate to acetone extracts were able to kill *E. coli* 25922. None of the biologically synthesized gold nanoparticle conjugates were able to exhibit minimum bactericidal concentration. Therefore, chemically synthesized gold nanoparticles are good for bactericidal activity compared to biologically synthesized gold nanoparticles. Our findings showed that the tuber extracts exhibited strong antimicrobial activity with the smallest MIC of 0.06 mg/ml which was smaller than the concentration of 1 mg/ml previously reported (Vatsos and Rebours, 2015).

The synergistic effects of gold nanoparticles conjugated to plant extracts were investigated against selected pathogenic bacteria using microdilution assay and the effects evaluated by determination of the FICI. Only 8 synergies (26.7%) , 12 additive (40%) and 10 antagonisms (33.3%) were observed from both biological and chemical synthesized gold nanoparticles conjugated *P. grandiflora* tubers extracts. In 2007, Rosato *et al*., reported synergistic interactions of gold nanoparticles conjugated to norfloxacin against *Staphylococcus aureus*, *Streptococcus mutans*, *E. coli* ATCC 25922 and *P. aeruginosa*. These synergistic activities of conjugates

suggest that it might be possible to reduce the viability of bacterial strains at lower antibiotic concentrations

6.6 CONCLUSION

In conclusion, this study has presented evidence of the antibacterial effects of *P. grandiflora* tuber extracts conjugated with gold nanoparticles and their synergistic capacity against selected pathogenic bacteria. Synergistic effects was observed against *E. coli*, *K. pneumonia* and MSSA when gold nanoparticles conjugated to *P. grandiflora* tuber extracts. Hence, all conjugates with synergistic effects are claimed to be responsible for improving effectiveness of many extracts and conventional antimicrobial drugs.

6.7 *REFERENCES*

Abtahi, S.M.H., (2013). Synthesis and characterization of metallic nanoparticles with photoactivated surface chemistries (Doctoral dissertation, Virginia Tech).

Agostoni, V., Horcajada, P., Noiray, M., Malanga, M., Aykaç, A., Jicsinszky, L., Vargas-Berenguel, A., Semiramoth, N., Daoud-Mahammed, S., Nicolas, V. and Martineau, C., (2015). A “green” strategy to construct non-covalent, stable and bioactive coatings on porous MOF nanoparticles. *Scientific reports*, 5, p.7925.

Bhumkar, D.R., Joshi, H.M., Sastry, M. and Pokharkar, V.B., (2007). Chitosan reduced gold nanoparticles as novel carriers for transmucosal delivery of insulin. *Pharmaceutical research*, 24(8), pp.1415-1426.

Cai, F., Li, J., Sun, J. and Ji, Y., (2011). Biosynthesis of gold nanoparticles by biosorption using *Magnetospirillum gryphiswaldense* MSR-1. *Chemical engineering journal*, 175, pp.70-75.

Gannimani, R., Ramesh, M., Mtambo, S., Pillay, K., Soliman, M.E. and Govender, P., (2016). γ-Cyclodextrin capped silver nanoparticles for molecular recognition and enhancement of antibacterial activity of chloramphenicol. *Journal of inorganic biochemistry*, 157, pp.15-24.

Grace, A.N. and Pandian, K., (2007). Antibacterial efficacy of aminoglycosidic antibiotics protected gold nanoparticles—A brief study. Colloids and Surfaces A: *Physicochemical and Engineering Aspects*, 297(1-3), pp.63-70.

Gu, H., Ho, P.L., Tong, E., Wang, L. and Xu, B., (2003). Presenting vancomycin on nanoparticles to enhance antimicrobial activities. *Nano letters*, 3(9), pp.1261-1263.

Iravani, S., (2011). Green synthesis of metal nanoparticles using plants. *Green Chemistry*, 13(10), pp.2638-2650.

Lengke, M.F., Fleet, M.E. and Southam, G., (2007). Biosynthesis of silver nanoparticles by filamentous cyanobacteria from a silver (I) nitrate complex. *Journal of Nanotechnology*, 23(5), pp.2694-2699.

Leopold, L.F., Tódor, I.S., Diaconeasa, Z., Rugină, D., Ştefancu, A., Leopold, N. and Coman, C., (2017). Assessment of PEG and BSA-PEG gold nanoparticles cellular interaction. Colloids and Surfaces A: *Physicochemical and Engineering Aspects*, 532, pp.70-76.

Mitra, P., Chakraborty, P.K., Saha, P., Ray, P. and Basu, S., (2014). Antibacterial efficacy of acridine derivatives conjugated with gold nanoparticles. *International journal of pharmaceutics*, 473(1-2), pp.636-643.

Mukherjee, P., Senatipati, S., Mandal, D., Ahamad, A. and Kumar, M., (2001). Extracellular synthesis of goldnanoparticles by fungus *F. oxysporum*. *Journal of Nanotechnology*. Rev, 24, pp.12-16.

Pelgrift, R.Y. and Friedman, A.J., (2013). Nanotechnology as a therapeutic tool to combat microbial resistance. *Advanced drug delivery reviews*, 65(13-14), pp.1803-1815.

Rosato, A., De Laurentis, N.V.N., Armenise, D., Millilo, M.A., (2007). Antibacterial effect of some essential oils administered alone or in combination with Norfloxacin. *Phytomedicine*, 14: 727-732. 10.1016.

Shrivastava, S., Bera, T., Roy, A., Singh, G., Ramachandrarao, P., and Dash, D. (2007). Characterization of enhanced antibacterial effects of novel nanoparticles.*Nanotechnology*, 18, 103e112.

Southam, G. and Beveridge, T.J., (1996). The occurrence of sulfur and phosphorus within bacterially derived crystalline and pseudocrystalline octahedral gold formed in vitro. *Geochimica et Cosmochimica Acta*, 60(22), pp.4369-4376.

Sun, Y. and Xia, Y., (2002). Shape-controlled synthesis of gold and silver nanoparticles. *Science*, 298(5601), pp.2176-2179.

Vatsos, I.N. and Rebours, C., (2015). Seaweed extracts as antimicrobial agents in aquaculture. *Journal of applied phycology*, 27(5), pp.2017-2035.

CHAPTER SEVEN: FUNCTIONALIZATION OF PENICILLIN, VANCOMYCIN AND AMPICILLIN WITH *PYRENACANTHA GRANDIFLORA* BAILL AND SILVER NANOPARTICLES

7.1 SUMMARY

BACKGROUND: The continuing rise in microbial drug resistance has led to widespread problems in the treatment of bacterial infections. Of particular concern are those illnesses caused by *Escherichia coli*, *Klebsiella pneumoniae* and methicillin-resistant *Staphylococcus aureus* (MRSA), which are responsible for a number of hospital- acquired infections, clinical complications, and deaths in some parts of the world. Some antibiotics have lost their efficacy over common infections and this has led to search on developing solutions to this problem by creating new antibiotics and chemically altering existing ones for a better control of infectious diseases.

OBJECTIVE: In this scenario we sought to functionalize penicillin, ampicillin and vancomycin since these are now less effective against hospital and community acquired infections caused by pathogens such as *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella Pneumonia*.

MATERIALS AND METHODS: *Pyrenacantha grandiflora* tubers extracts were conjugated with ampicillin, penicillin, vancomycin and silver nanoparticles. Conjugations were confirmed by formation of new functional groups that were identified by Fourier transmission infrared spectroscopy (FTIR). Conjugated extracts were evaluated for antibacterial activity with agar diffusion assay. Minimum inhibitory concentrations were then determined using the microdilution assay. Minimum bactericidal concentrations as well as fractional inhibition concentration index were also determined.

RESULTS: FTIR analysis indicated functional bio-molecules associated with antibiotics conjugated plant extracts and silver nanoparticles such as formation of C-H group and carbonyl

(C=O) group, however, -OH carbonyl and C≡C group was also observed. Well diffusion assay showed that the activity of ampicillin is improved when conjugated with silver nanoparticles against *K. pneumonia* and *E. coli*. Vancomycin shows improvement of activity when conjugated to silver nanoparticles against *K. pneumonia*. From the MIC results, penicillin was improved by acetone extracts and vancomycin shows to be more effective when conjugated with silver nanoparticles and water extracts.

CONCLUSION: The overall results indicate that *P. gandiflora* and conjugates are medicinally important and can be used to improve the activity of existing antibiotic that have become less effective on their own.

Keywords: Antibacterial, antibiotics, silver nanoparticles, plant extract, agar diffusion assay, Minimum fungicidal concentration

7.2 INTRODUCTION

The occurrence of the infectious diseases caused by different pathogenic bacteria has become a challenge the world is facing, and the development of antibiotic resistance is threatening our capacity to treat regular ailments. Hence bringing about protracted diseases, disability and death (WHO, 2016). Bacterial resistance is of considerable economic importance, and in combination with the undesirable side effects of some synthetic compounds, it becomes necessary and imperative to search for new and cheaper molecules with lesser side effects (Dzotam *et al*., 2018).

Multi drug resistant strains of *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae* cause health care associated infections in hospitals and have evolved drug resistance to many of the established antimicrobial compounds, on the market at present (Brown *et al*., 2012). For example, mutation in DNA gyrase enzyme results in resistance towards quinolones in *E. coli* and *S. pneumonia* (Pan *et al*., 2001). *Staphylococcus aureus* has developed resistance against vancomycin (Sieradzki, 2006) and mutations affecting RNA polymerase β subunit have resulted in *E. coli* resistance towards rifampicin. The loss of effectiveness of commonly used antibiotics such as penicillin and other β-lactam drugs further add to the dilemma, calling for the immediate need for improvements in drug design, discovery, and delivery.

In the present study, medicinal plants and silver nanoparticles are emerging to address the challenge due to their efficacy as antimicrobial agents. In the recent times, silver nanoparticles (AgNPs) have shown profound impact on food, cosmetics, and beverage (Saravanan *et al*., 2018). A plenty of studies have proven that AgNPs possess outstanding antimicrobial, anti-platelet, anti-inflammatory, anti-angiogenic, anticancer and antiviral activities with less toxicity and enhanced biodegradability and bioavailability in industrial applications (Subbaiya *et al*., 2017; Barabadi *et al*., 2017; Saratale *et al*., 2017 and Ramkumar *et al*., 2017).

In most studies, an increase in antimicrobial activity was reported after conjugation of antibiotic to nanoparticles. Hence, AgNPs were reported to improve antimicrobial activity of ampicillin (Abhishek and Hemlata, 2014). Silver nanoparticle was also reported to have antibacterial activity when synthesized using plant extract (Yazdi *et al*., 2018). This study aimed to evaluate antibacterial activity of *Pyrenacantha grandiflora* Baill extracts when conjugated with silver nanoparticles and antibiotics (vancomycin, ampicillin and penicillin) against pathogenic bacteria (*Staphyloccocus aureus, Escherichia coli, Klebsiella pneumoniae*) in order to achieve synergistic effect and thus offer advantages such as dosage compliance, minimizing toxicity and overcoming drug resistance when compared to the parent counterparts (Wang *et al*., 2007).

7.3 MATERIAL AND METHODS

7.3.1 Used microorganism, growth conditions and antibiotics

The microorganisms that were used in this study included methicillin-resistance *Staphylococcus aureus* (ATCC 25923), *Beta* lactamase producing *Klebsiella pneumoniae* (ATCC 700603) and *Beta* lactamase producing *Escherichia coli* (ATCC 35218). These bacterial strains were maintained on mannitol salt agar, MacConkey agar and nutrient agar respectively, all media were purchased from Rochelle Chemicals (SA). An inoculum of each bacterial strain was suspended in 5 ml of Mueller Hinton broth (Rochelle chemicals, SA) and incubated 3 hours at 37°C. The cultures were diluted with Mueller Hinton Broth and adjusted to give a concentration of bacterial cells equivalent to a McFarland 0.5 standard prior to the antibacterial testing. The following antibiotics were used in this study: Penicillin (Sigma, Germany), Vancomycin (Fluka chemie, Denmark) and Ampicillin (Sigma-Aldrich, USA).

7.3.2 Conjugation of antibiotics and plant extract

Plant extracts were conjugated to the antibiotics as previously described by (Tom *et al*., 2004). The antibiotic solutions were filter-sterilized using 22 µm filter and refrigerated at 4°C. *Pyrenacantha grandiflora* tubers compounds were extracted using acetone, water and methanol as solvents. Each of the plant extracts (10 mg/ml) were mixed with 10 mg/ml of each antibiotics. The mixture was incubated at 30°C in a rotary shaker for 6 hours and then stored at 4°C for further analysis.

7.3.3 Conjugation of silver nanoparticles with antibiotics

Antibiotic-plant extract conjugations were prepared by dissolving the respectiveantibiotic (penicillin, vancomycin, and ampicillin) in distilled water to the concentration of 10 mg/ml. The antibiotic solutions were filter-sterilized using 22 µm filter and refrigerated at 4°C. The combined reducing property of tri-sodium citrate and 10 mg/ml of antibiotics were used to reduce silver nitrate ($AgNO_3$) (Ansari *et al*., 2011) in order to prepare the conjugates. The conjugates were then stored at 4°C for further analysis.

7.3.4 Conjugation of antibiotics, plant extracts, and silver nanoparticles

Antibiotic-silver nanoparticle-plant extract conjugations were done by mixing 10 mg/ml of respective antibiotics (Penicillin, Vancomycin, and Ampicillin), 10 mg/ml of plant extract (methanol, acetone and water extract) and 1 mM Silver nitrate (Sigma, USA) to make 50 ml solution. The mixtures were incubated at 30°C for 30 min. The conjugates were cooled and stored at 4°C for further analysis. The formation of the conjugates were denoted by the formation of intense brown color from colorless. The bottle carrying the mixture was covered with a foil and

stored in the dark to avoid the photo-activation of silver nitrate under static conditions. Methanol, acetone and water crude extract as well as 1mM silver nitrate and antibiotics solution were measure with UV-Vis Spectrometer at a range of the 200-800 nm. The silver nanoparticles were purified by centrifugation at maximum speed of 13000 g for 1 hour, followed by washing with distilled water and resuspended in distilled water.

7.3.5 Characterization of conjugated antibiotics

All conjugates were analyzed using Fourier- transmission electron microscopy (FTIR) in a range of 400-4000 cm^{-1} to detect various functional groups formed after conjugation that may be responsible for biological activities. Conjugates (500 µl) were placed on the sample chamber of FTIR spectrophotometer and the spectra were recorded in the scan range of 400–4000 cm^{-1} with a resolution of 4 cm^{1} on a Nicolet Avatar 330 FTIR spectrometer.

7.3.6 Well diffusion assay

The antibacterial activities of the conjugates were determined by well diffusion method. The zone of inhibitions was recorded in millimeter (mm). Briefly, bacterial suspensions of *E. coli*, *K. pneumonia, and S. aureus* were prepared with the turbidity of 0.5 McFarland. Mueller-Hinton agar plates were inoculated with that bacterial suspension. Wells with a diameter of 6 mm were cut using a cork borer and filled with 30 µl of the conjugated antibiotics and reference samples were non conjugated plant extracts, antibiotics and silver nanoparticles. Distilled water was used as a negative control and gentamycin as positive control. Plates were incubated for 24 hours at 37°C. After incubation, the growth inhibition zone diameters were measured.

7.3.7 Microdilution assay

The minimum dilution of all antibiotics plus conjugates samples that inhibits the growth of the microorganism were denoted as minimum inhibitory concentration (MIC) (Samie et al., 2005). Distilled water was used as negative control and ciprofloxacin was used as positive control. After adding INT (iodo-nitro tetrazolium), the results were read by observing the color change and determining the MIC. All samples that showed activity (reduced or no color change) were inoculated again in the agar plate and incubated overnight to determine the minimum bactericidal concentration (MBC).

7.3.8 Determination of Fractional Inhibition Concentration Index (FICI)

Determination of the mutual influence of silver nanoparticles and plant extracts in antibiotics were done using Fractional Inhibition Concentration Index by the following formula:

$$FICI = \frac{MIC\ of\ AB}{MIC\ of\ A} + \frac{MIC\ of\ AB}{MIC\ of\ B}$$

Where AB represent a combination of *P. grandiflora* tubers extracts (A) and antibiotic (B). Results were interpreted as synergy (FICI≤0.5), antagonism (FICI>4) and no interaction or additive (FICI>0.5-4.0).

7.4 RESULTS

7.4.1 Antibiotics and plants extrac

7.4.1.1 FTIR analysis of ampicillin conjugated plant extracts

The ampicillin-acetone extracts exhibited characteristic bands at 3368 (O-H Stretching), 2068 (C≡C Stretching), 1697 (C=C isolated), 1638 (C=C conjugated), 1383 (C-H bending), 1368 (C-H bending), 1234 (C-O Stretching) and 1091 (O-H bending) as illustrated in Figure 7.1. Bands at 1383 (C-H bending) and 1234 (C-O Stretching) were not observed in unconjugated ampicillin nor acetone (**Figure 7.1**) While bands at 2953 (C-H stretching) and 1111 (C-O Stretching) were not observed in unconjugated ampicillin nor methanol extracts.

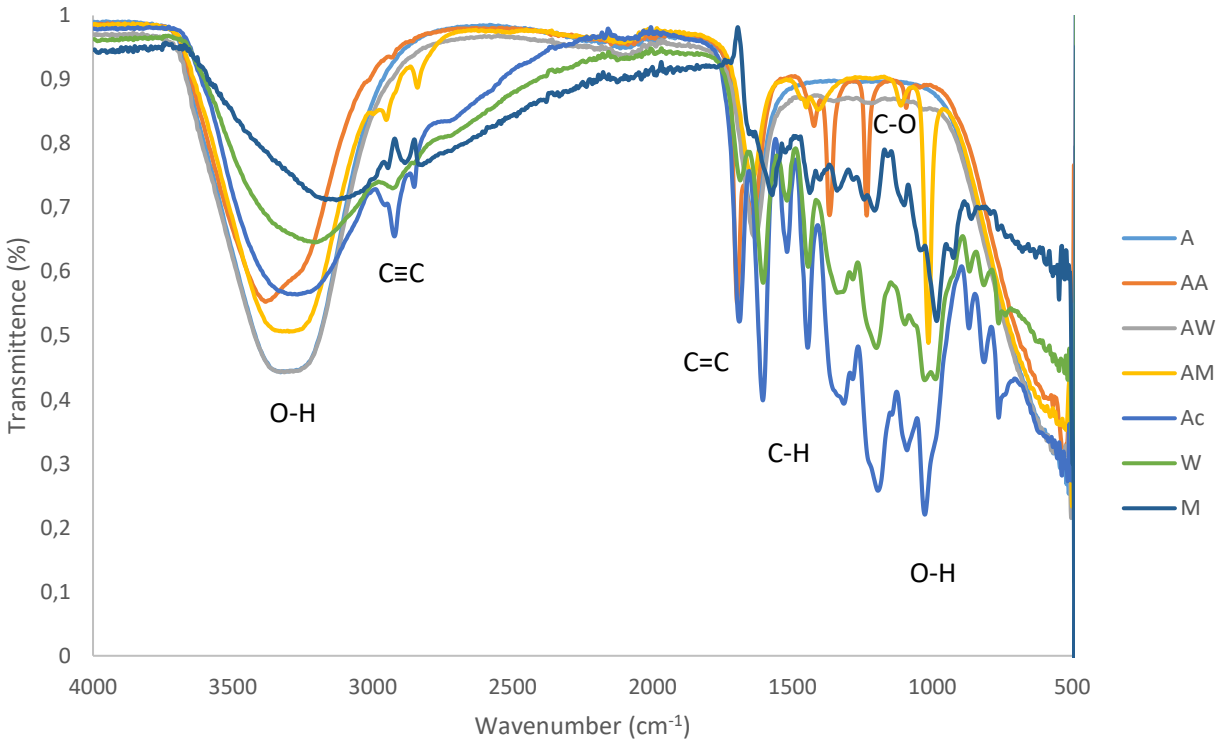


Figure 7.1: FTIR spectrum of ampicillin conjugates with acetone, water, methanol extracts of *P. grandiflora* as a reference sample, where A= ampicillin, AA= ampicillin-acetone extract, AW= ampicillin-water extract, AM= ampicillin-methanol extract, Ac= acetone extract, M= methanol extract and W= water extract.

7.4.1.2 FTIR analysis of penicillin conjugated plant extracts

The FTIR spectrum of penicillin plants conjugates (**Figure 7.2**) illustrated absorption bands 3388 (O-H Stretching), 3272 (C-H Stretching), 3004 (C-H Stretching), 2101 (C≡C Stretching), 1701 (C=O group), 1644 (C=C isolated), 1419 (C-H stretching), 1360 (C-H bending), 1232 (C-O Stretching), 1093 (O-H bending). Only 1232 (C-O Stretching) and 1419 (C-H stretching) functional groups were formed in penicillin-acetone extracts whereas in penicillin methanol extracts 2988 and 2839 (C-H stretching) were observed as new functional groups.

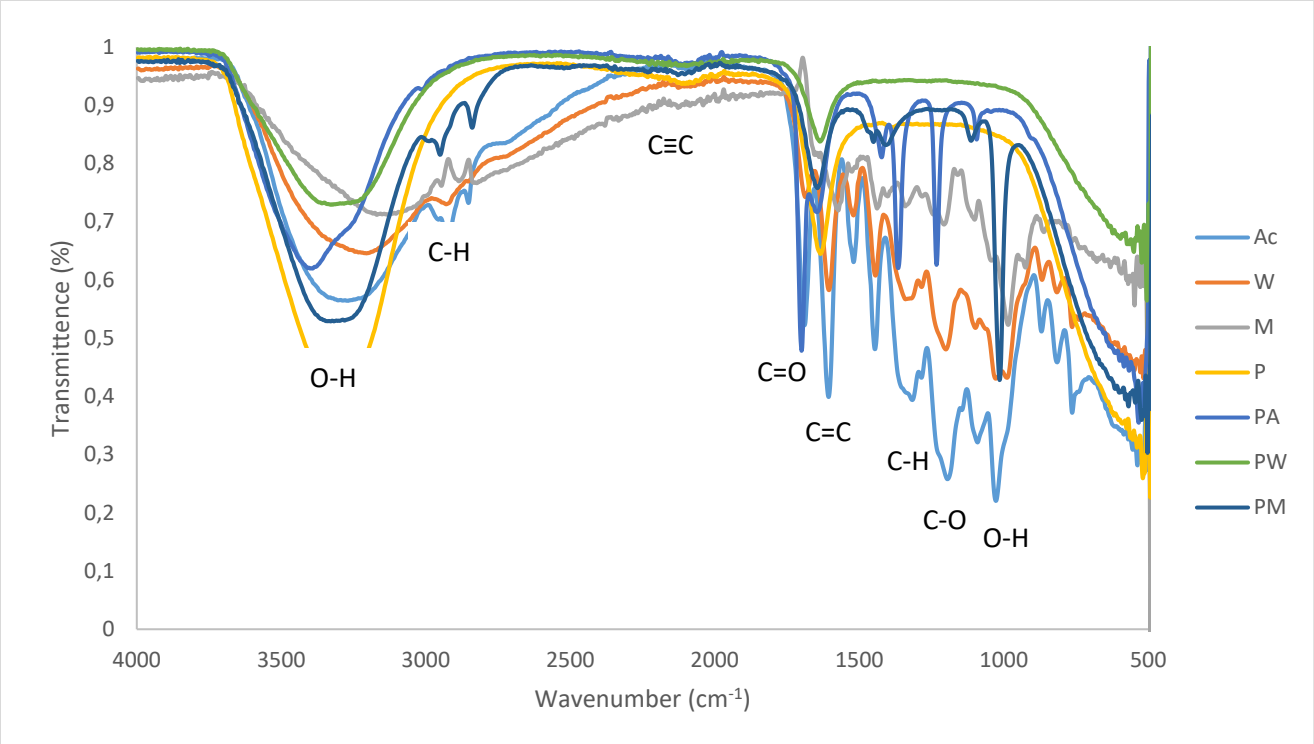


Figure 7.2: FTIR spectrum of penicillin conjugated with acetone, water and methanol extracts of *P. grandiflora* where, P= penicillin-methanol extract, PA= penicillin-acetone extract, PW= penicillin-water extract, Ac= acetone extract, M= methanol extract and W=water extract.

7.4.1.3 FTIR analysis of vancomycin conjugated plant extracts

Vancomycin-plant extracts conjugates exhibited characteristic bands at 3363 (O-H Stretching), 3255 (C-H Stretching), 2101 (CC Stretching), 1691 (C=C isolated), 1644 (C=C isolated), 1419 (C-H Stretching), 3662 (C-H bending), 1234 (C-O Stretching) and 1093 (O-H bending). 3255 and 1491(C-H stretching) and1234 (C-O stretching) were the new functional groups formed in vancomycin-acetone extract conjugates (**Figure 7.3**). Hence 2831 (C-H Stretching), 1401 (C=C isolated) and 1009 (C-O Stretching) bands were observed in vancomycin-methanol extracts, but not present in unconjugated vancomycin nor water extracts.

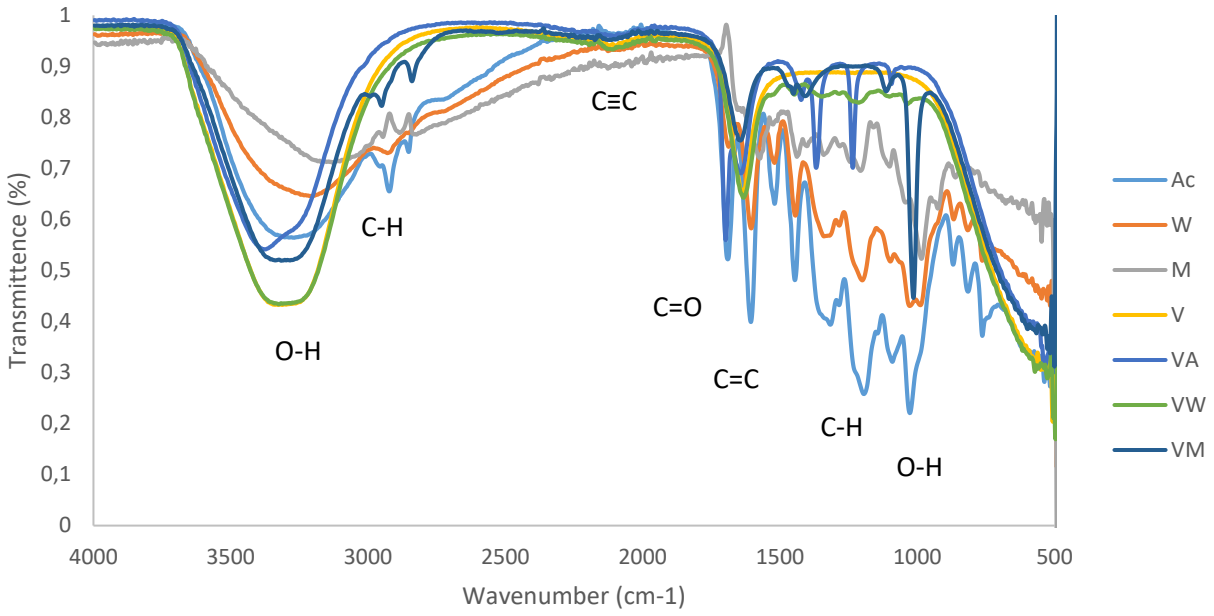


Figure 7.3: FTIR spectrum of vancomycin conjugated with acetone, water extract and methanol extracts of *P. grandiflora* where, V= vancomycin, VA= vancomycin-acetone extract, VW= vancomycin-water extract, VM= vancomycin-methanol extract, A= acetone extract, M= methanol extract and W=water extract.

7.4.2 Antibiotics and silver nanoparticles

From the FTIR spectrum, percent transmission was plotted against wavenumber and the greater the amount of light absorbed by the sample, the smaller the percent transmittance. Different functional groups corresponding to absorbance peaks in the range of 3000–3360 (O-H stretching), 2800–3000 (C≡C stretching) and 1603-1697 (C=C conjugated) cm^{-1} were observed in almost all antibiotics conjugated silver nanoparticles. However, the difference was observed in the absorbance and peaks at different wavenumbers on same functional groups (**Figure 7.4).** No new functional group was observed when silver nanoparticles were conjugated with penicillin and ampicillin. Vancomycin showed two new C≡C functional group. The peaks are known to be associated with stretching vibrations of hydroxyl groups (O-H) in alcohols or phenolic compounds,

CH2 and CH3 functional groups; C=C groups of aromatic compounds or C=O groups of carboxylic acids.

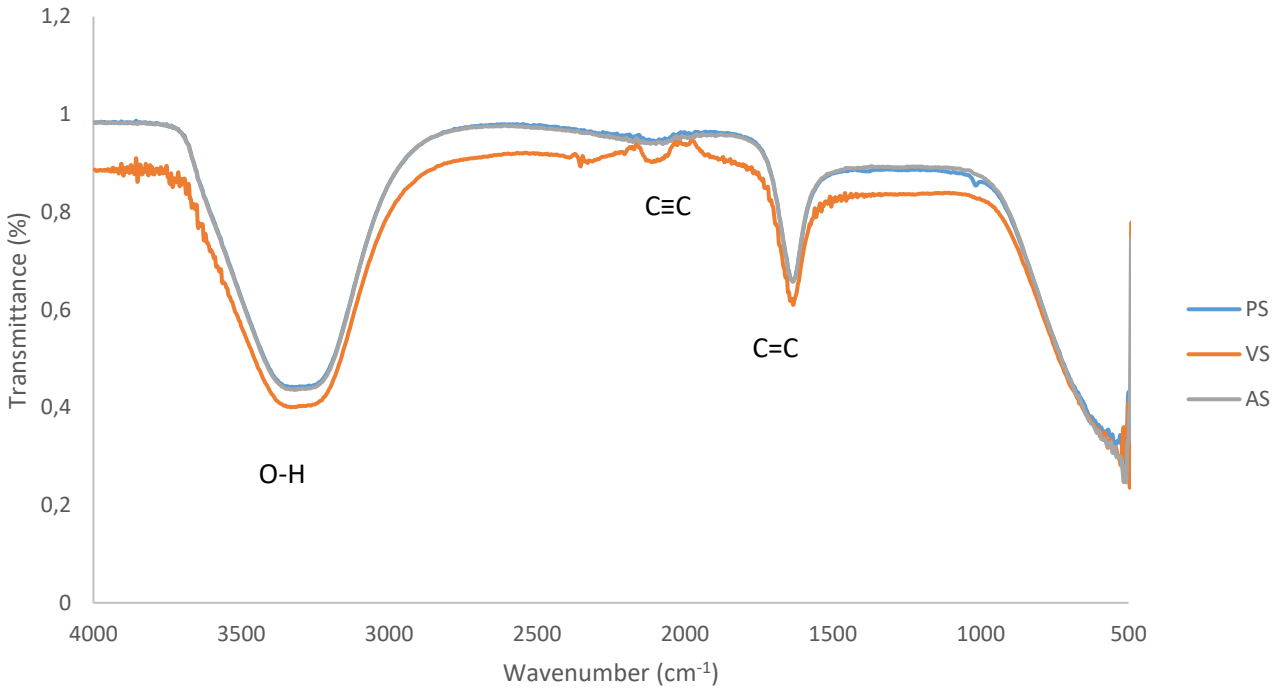


Figure 7.4: FTIR spectrum of antibiotics conjugated silver nanoparticles where: PS (penicillin conjugated silver nanoparticles); VS (vancomycin conjugated silver nanoparticles) and AS (ampicillin conjugated silver nanoparticles).

7.4.3 Conjugation of Antibiotics with plant extract and nanoparticles

7.4.3.1 Analysis of ampicillin conjugated silver nanoparticle and plant extracts

When ampicillin was conjugated with silver nanoparticles and plant extracts it exhibited characteristic bands at 3350 (O-H Stretching), 3247 (C=C isolated), 2028 (CC Stretching), 1697 (N-H Stretching), 1644 (C=C isolated), 1419 (C-H Stretching), 1362 (C-H bending), 1234 (C-O Stretching), 1095 (O-H bending) and 1020 (C-O Bending) (**Figure 7.5**). Newly formed functional groups from the conjugation included 1234 (O-H str) and 1095 (Bending) from ampicillin-silver

nanoparticles-acetone extracts (ASA), 2839 (C-H str) and 1111(C-O str) from ampicillin-silver nanoparticles-methanol extracts and 1113 (C=O Str), 1103 (C-O Str) and 1020 (O-H bending) from ampicillin-silver nanoparticles-water extracts.

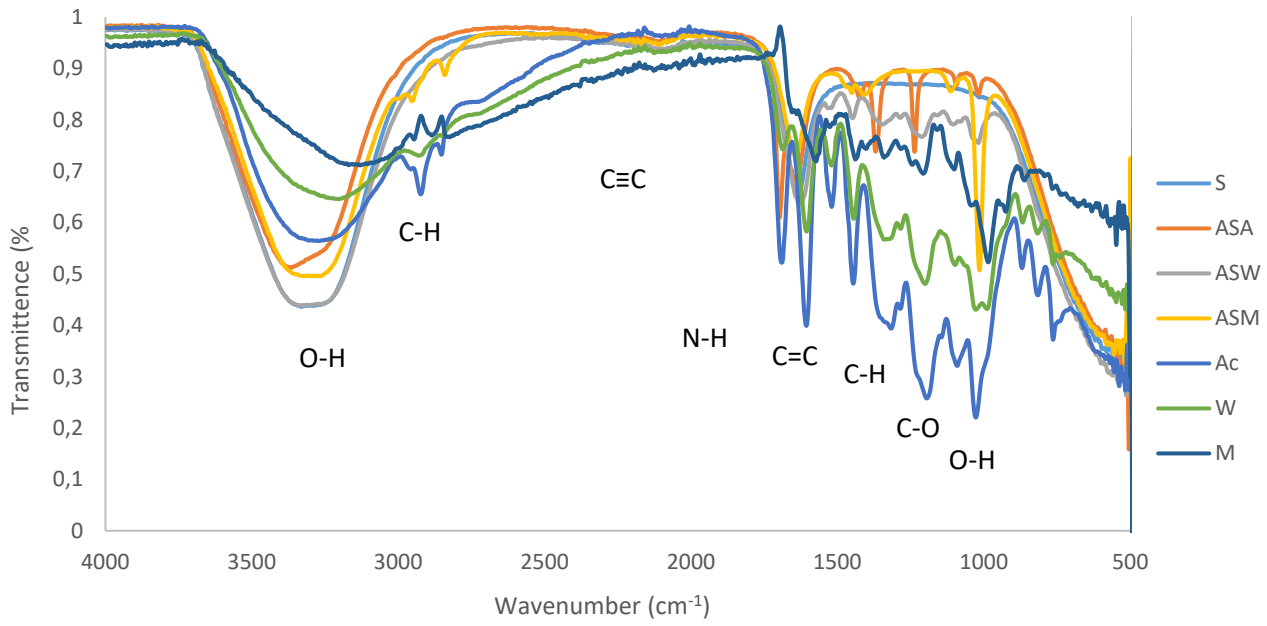


Figure 7.5: FTIR spectra of ampicillin conjugated silver nanoparticles and plant extracts where: S= silver nanoparticles, ASA= ampicillin-silver nanoparticles- acetone extract, ASW= ampicillin-silver nanoparticles- water extract, ASM= ampicillin-silver nanoparticles - methanol extract, A= acetone extract, M= methanol extract and W=water extract.

7.4.3.2 Analysis of penicillin conjugated silver nanoparticle and plant extracts

Penicillin conjugated silver nanoparticles and plant extracts exhibited characteristic bands at 3370 (O-H Stretching), 2082 (C≡C Stretching), 1699 (C=C isolated), 1644 (C=C isolated), 1423 (C-H bending), 1368 (C-H Bending), 1234 (C-O Stretching), 1095 (O-H bending) and 1020 (C-O Bending) (**Figure 7.6**). A newly formed functional groups from conjugation included 1234 (C-O Stretching) and 1423 (C-H bending) from penicillin conjugated silver nanoparticles and acetone extract, 2839 and 1409 (C-H str) from penicillin conjugated silver nanoparticles and methanol

extract and no new functional group from penicillin conjugated silver nanoparticles and water extracts.

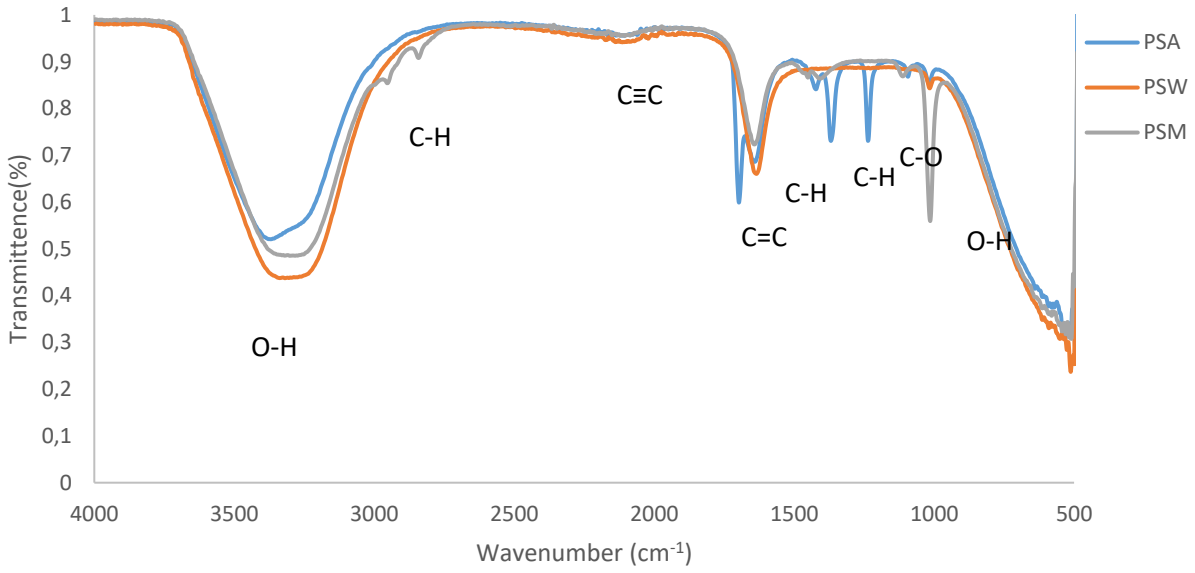


Figure 7.6: FTIR spectrum of penicillin conjugated silver nanoparticles and plant extracts where, PSA= penicillin-silver nanoparticles-acetone extract, PSW= penicillin-silver nanoparticles- water extract, PSM= penicillin-silver nanoparticles- methanol extract, S= silver nanoparticles, A= acetone extract, M= methanol extract and W=water extract.

7.4.3.3 Analysis of vancomycin conjugated silver nanoparticle and plant extracts

Vancomycin conjugated silver nanoparticles and plant extracts exhibited characteristic bands at 3372 (O-H Stretching), 2082 (C≡C Stretching), 1697 (C=C isolated), 1644 (C=C isolated) and 1011 (C-O Stretching) which were also observed in unconjugated vancomycin, plant extract or silver nanoparticles (**Figure 7.7**). Newly formed functional group included 1383 (C-H Bending), 1368 (C-H Stretching), 1238 (C-O Stretching) and 1095 (O-H Bending) from vancomycin conjugated silver nanoparticles and acetone extracts, 2841, 1454 and 1401 (C-H str) and 1113 (C-O str) from vancomycin conjugated silver nanoparticles and methanol extracts and no new

functional group was observed with vancomycin conjugated silver nanoparticles and water extracts.

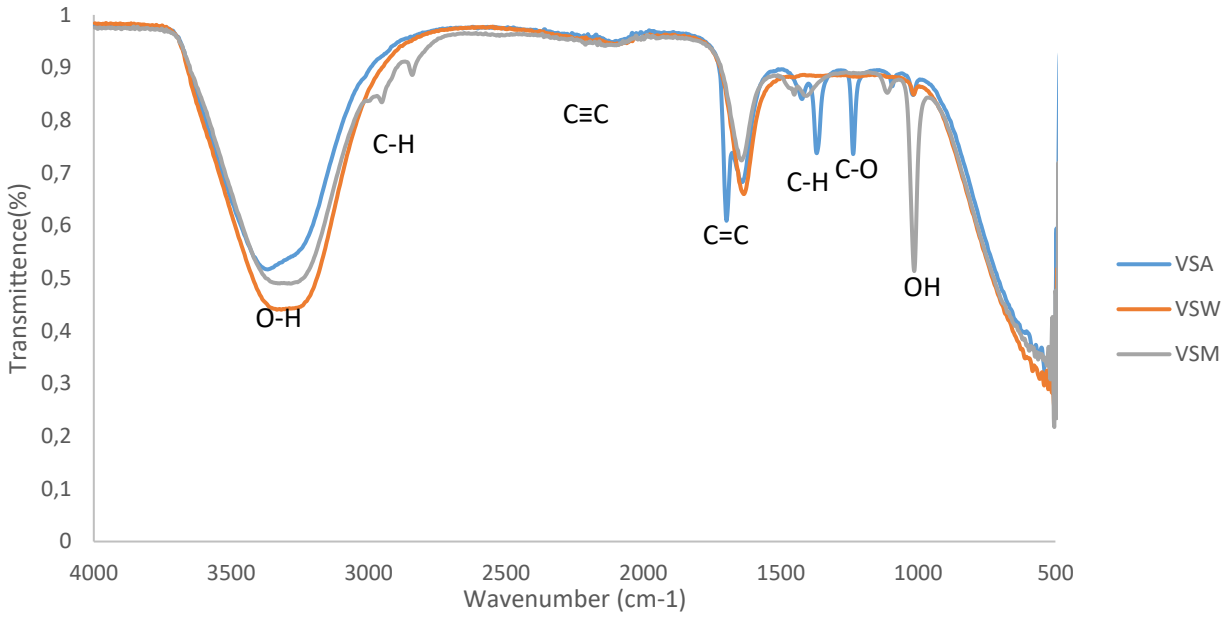


Figure 7.7: FTIR spectrum of vancomycin conjugated silver nanoparticles and plant extracts where, VSA= vancomycin-silver nanoparticles- acetone extract, VSW=Vancomycin-silver nanoparticles-water extract, Vancomycin-silver nanoparticles- Methanol extract, S=silver nanoparticles, A= acetone extract, M= methanol extract and W=water extract

7.4.4. Well diffusion assay

7.4.4.1 Antibacterial activity of ampicillin conjugated silver nanoparticle and plant extracts

Ampicillin was conjugated with silver nanoparticles and plant extracts and their antibacterial activity was evaluated by the well diffusion assay. Conjugated ampicillin was active against all tested bacteria. Antibacterial activity was observed when ampicillin conjugated to the silver nanoparticles and acetone extracts (ASA) against *K. pneumonia* with the highest zone of growth inhibition of 18 mm (**Figure 7.8**). However, very low antibacterial activity was observed when

ampicillin conjugated with silver nanoparticle and water extract against *K. pneumonia* with the zone of growth inhibition of 6 mm.

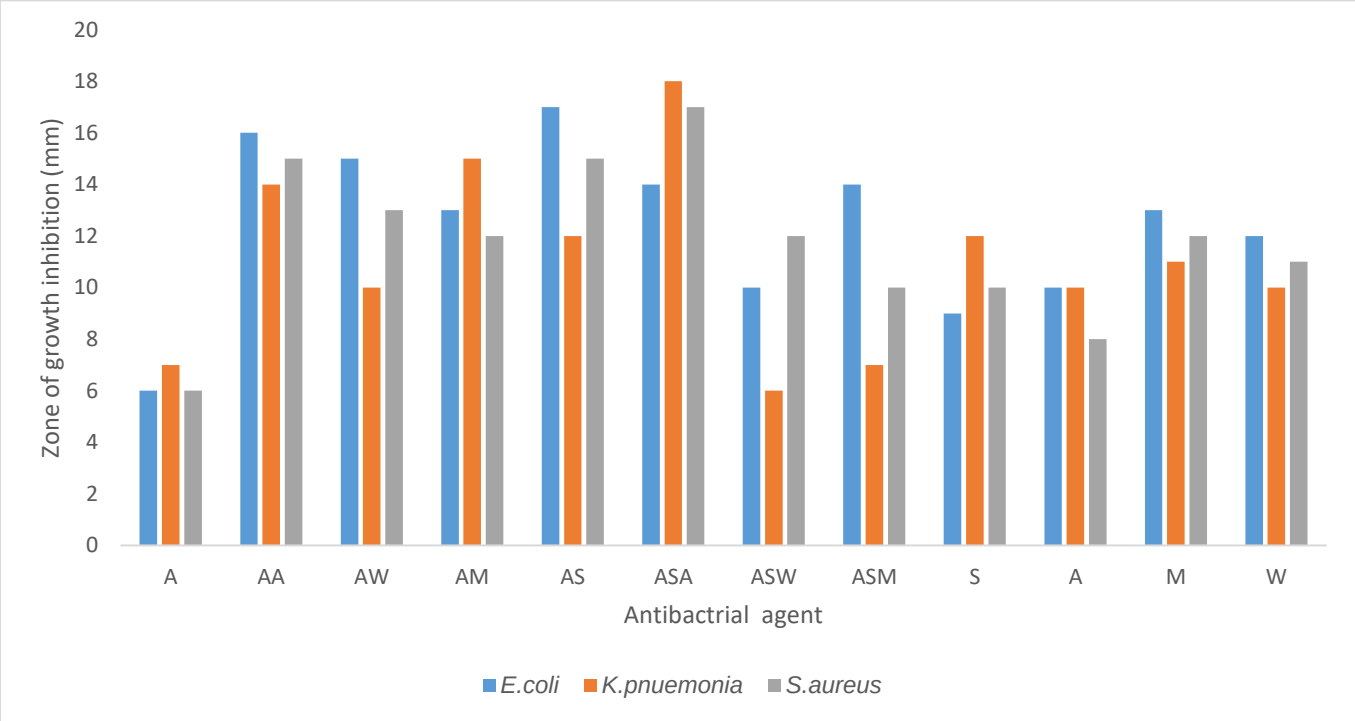


Figure 7.8: Antibacterial activity of ampicillin conjugated silver nanoparticles and plant extracts against three bacterial where: A= ampicillin, AA= ampicillin-acetone extract, AW= ampicillin-water extract, AM= ampicillin-methanol extract, AS= ampicillin-silver nanoparticles, ASA= ampicillin-silver nanoparticles-acetone extract, SW= ampicillin-silver nanoparticles-water extract, ASM= ampicillin-silver nanoparticles-methanol extract, S- silver nanoparticles, A= acetone extract, M= methanol extract and W=water extract.

7.4.4.2 Antibacterial activity of penicillin conjugated silver nanoparticles and plants extracts

Antibacterial activity was studied against three pathogenic resistant bacterial strains with well diffusion assay. Interesting results were observed with silver nanoparticles conjugated with antibiotic and plant extract especially the acetone extract. The antibacterial activity of conjugated penicillin are shown in **Figure 7.9**. The activity of penicillin shows a massive increase when conjugated with acetone extract and silver nanoparticle against *E. coli* and *S. aureus* with the

highest zone of growth inhibition of 18 mm. Most conjugated penicillin was most active against *E. coli* while MRSA shows resistance against penicillin-water extract and in penicillin-water extract-silver nanoparticles.

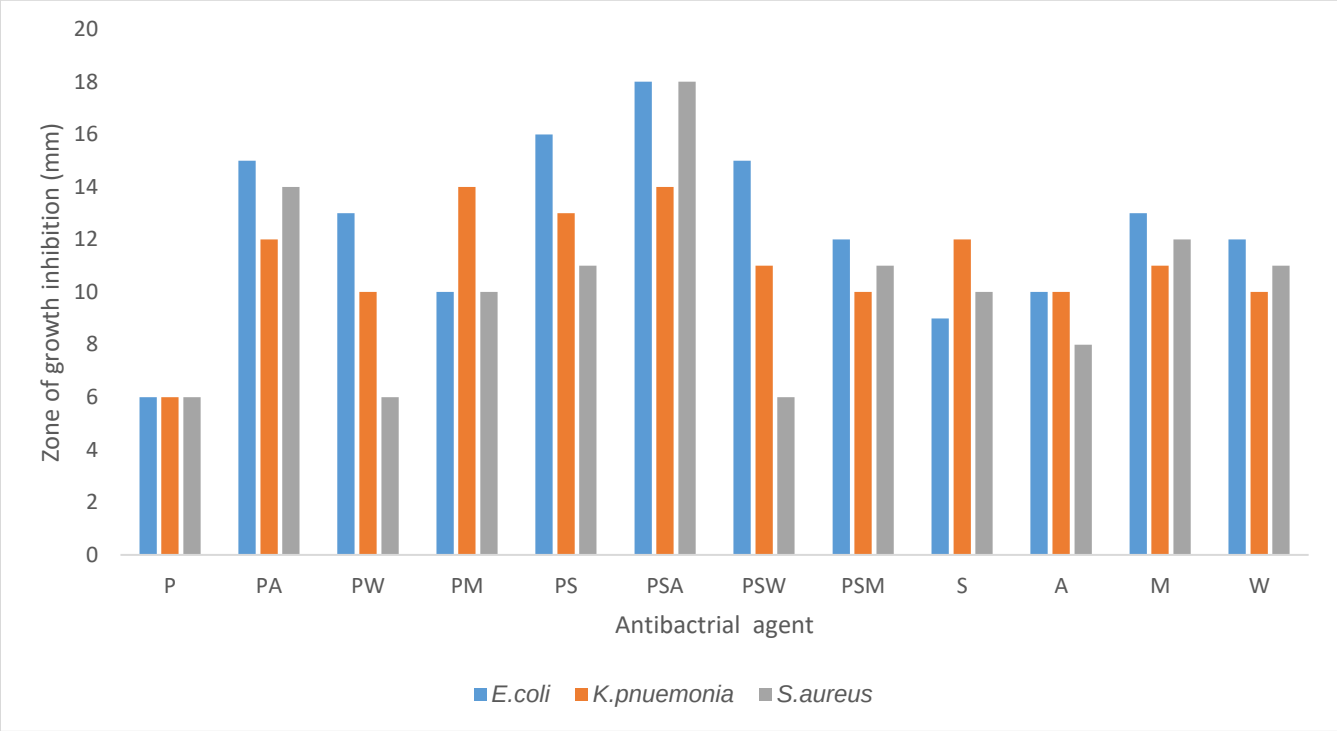


Figure 7.9: Antibacterial activity of penicillin-conjugated silver nanoparticles and plant extracts where, P= penicillin-methanol extract, PA= penicillin-acetone extract, PW= penicillin-water extract, PS= penicillin-silver nanoparticles, PSA= penicillin-silver nanoparticles-acetone extract, PSW= penicillin-silver nanoparticles-water extract, PSM= penicillin-silver nanoparticles- methanol extract, S= silver nanoparticles, A= acetone extract, M= methanol extract and W=water extract.

7.4.4.3 Antibacterial activity of vancomycin conjugated silver nanoparticles and plants extract

The highest activity was observed when vancomycin was conjugated with silver nanoparticles and acetone extract against *E. coli* with a zone of growth inhibition of 18 mm (**Figure 7.10**). Vancomycin-silver nanoparticles-water extract and vancomycin-water extract showed a minor increase of antibacterial activity of 7 mm and 8 mm respectively against *K. pneumonia*.

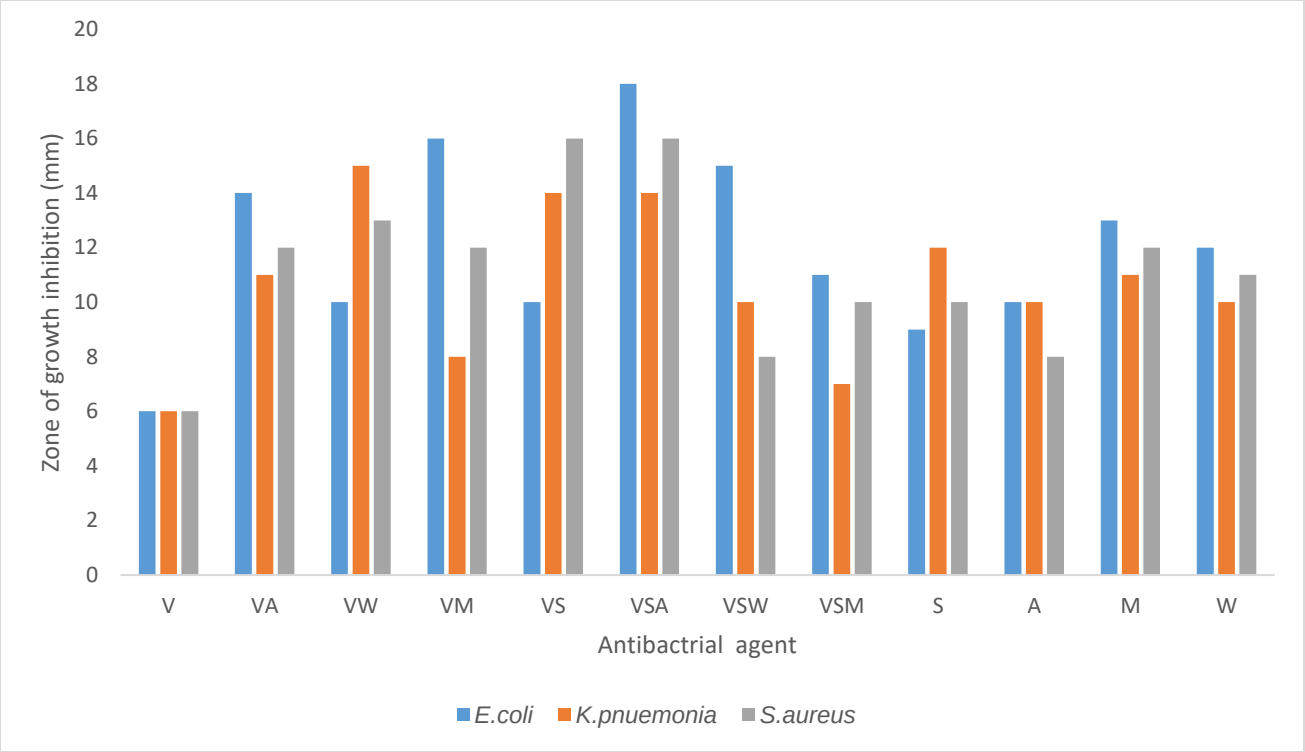


Figure 7.10: Antibacterial activity of vancomycin conjugated silver nanoparticles and plant extracts on three bacterial strain, Where: V= vancomycin, VA= vancomycin-acetone extract, VW= vancomycin-water extract, VM= vancomycin-methanol extract, VS= vancomycin-silver nanoparticles, VSA= vancomycin-silver nanoparticles- acetone extract, VSW= vancomycin-silver nanoparticles-water extract, VSM= vancomycin-silver nanoparticles-methanol extract, S=silver nanoparticles, A= acetone extract, M= methanol extract and W= water extract.

7.4.5 Microdilution assay

7.4.5.1 Minimum inhibitory concentration of conjugated ampicillin

Microdilution assay was used to determine the minimum inhibitory concentration of ampicillin conjugated plant extracts and silver nanoparticles. The concentration used range from 0.8-0.0063 mg/ml (**Table 7.1**). When ampicillin is conjugated with silver nanoparticles and plant extracts exhibit lowest MIC value of 0.0063 mg/ml against *K. pneumonia*. Ampicillin conjugated silver nanoparticles showed the lowest MIC value of 0.05 mg/ml was observed against *E.coli* and 0.2

mg/ml against *S. aureus*. However, ampicillin alone needs more than 0.8 mg/ml to inhibit the growth of *E. coli*, *K. pneumonia* or *S. aureus*.

Table 7.1: Minimum inhibitory concentration (MIC) of ampicillin conjugated plant extracts and silver nanoparticles.

Sample	*E. coli*	*K. pneumoniae*	*S. aureus*
A	0.8	0.8	0.8
AA	0.4	0.2	0.4
AW	0.8	0.4	0.2
AM	0.8	0.4	0.2
AS	0.05	0.8	0.2
ASA	0.4	0.4	0.4
ASW	0.8	**0.0063**	0.4
ASM	0.8	**0.1**	0.4

Key: A= ampicillin, AA= ampicillin-acetone extract, AW= ampicillin-water extract, AM= ampicillin-methanol extract, AS= ampicillin-silver nanoparticles, ASA= ampicillin-silver nanoparticles-acetone extract, ASW= ampicillin-silver nanoparticles- water extract, ASM= ampicillin-silver nanoparticles - methanol extract.

7.4.5.2 Minimum inhibitory concentration (MIC) of conjugated penicillin

Minimum inhibitory concentration of conjugated penicillin was determined by microdilution assay. The concentration used range from 0.8-0.0063 mg/ml (**Table 7.2**). Distilled water was used as a negative control and gentamycin was used as positive control. More than 0.8 mg/ml of penicillin was needed to inhibit the growth of *E. coli*, *K. pneumonia* and *S. aureus*. Lowest MIC values were observed in penicillin conjugated silver nanoparticles with 0.0125 mg/ml against *S. aureus.* Penicillin conjugated methanol and also with silver nanoparticles were effective in inhibiting the growth of *E.coli* at MIC values 0.1 and 0.025 mg/ml.

Table 7.2: Minimum inhibitory concentration (MIC) of penicillin conjugated plant extracts and silver nanoparticles.

Sample	*E. coli*	*K. pneumonia*	*S. aureus*
P	0.8	0.8	0.8
PA	0.4	0.2	0.4
PW	0.4	0.4	0.4
PM	**0.1**	0.4	0.4
PS	**0.05**	0.8	**0.0125**
PSA	0.4	0.4	0.8
PSW	0.8	**0.025**	0.8
PSM	**0.025**	0.8	0.8

Key: P= penicillin-methanol extract, PA= penicillin-acetone extract, PW= penicillin-water extract, PS= penicillin-silver nanoparticles, PSA= penicillin-silver nanoparticles-acetone extract, PSW= penicillin-silver nanoparticles-water extract, PSM= penicillin-silver nanoparticles-methanol extract.

7.4.5.3 Minimum inhibition concentration of conjugated vancomycin

Minimum inhibitory concentrations of conjugated vancomycin were tested against three pathogenic bacteria using microdilution assay. All the tests were conducted in duplicate. The MIC values obtained from vancomycin conjugated plant extracts and silver nanoparticles are shown in **Table 7.3**. The MIC of conjugated vancomycin was seen in the range of 0.8-0.0063 mg/ml (**Table 7.3**). The lowest concentration to inhibit the growth of *E.coli* amongst the conjugate was observed when vancomycin is conjugated with silver nanoparticles and methanol extracts with MIC value of 0.0125 mg/ml.

Table 7.3: Minimum inhibitory concentration (MIC) of vancomycin conjugated plant extracts and silver nanoparticles.

Samples	*E. coli*	*K. pneumonia*	*S. aureus*
V	0.8	0.4	0.8
VA	0.8	0.4	0.4
VW	0.8	0.4	0.4
VM	0.8	0.4	0.8
VS	0.2	**0.05**	**0.1**
VSA	0.4	0.4	0.4
VSW	0.8	0.4	0.4
VSM	**0.0125**	0.4	0.4

Key: V= vancomycin, VA= vancomycin-acetone extract, VW= vancomycin-water extract, VM= vancomycin-methanol extract, VS= vancomycin-silver nanoparticles, VSA= vancomycin-silver nanoparticles-acetone extract, VSW= vancomycin-silver nanoparticles-water extract, VSM= vancomycin-silver nanoparticles-methanol extract.

7.4.6 DETERMINATION OF FRACTIONAL INHIBITION CONCENTRATION INDEX (FICI)

7.4.6.1 Fractional inhibition concentration index of conjugated ampicillin

Determination of the mutual influence of antibiotics, silver nanoparticles and plant extracts in conjugate was done using fractional inhibition concentration index (FICI). Fractional inhibition concentration index was calculated on the results obtained from MIC and the results are recorded in **Table 7.4**. A total of seven samples of conjugated ampicillin were tested against three bacterial ATCC strains. Only 1 synergy (4.8%) was observed, 9 (42.8%) were additive and 11 (52.4%) were antagonism.

Table 7.4: Effect of conjugating ampicillin with plant extracts and silver nanoparticles.

Samples	*E. coli*	*K. pneumonia*	*S. aureus*
AA	2 (A)	2(A)	2(A)
AM	5 (N)	1.5(A)	**0.32 (S)**
AW	3(A)	3.99(A)	33(N)
AS	1(A)	17(N)	4.5(N)
ASA	5(N)	9(N)	9(N)
ASW	9.5(N)	1.134(A)	71.9(N)
ASM	13(N)	2.37(A)	9.14(N)

Key: A (Additive), N (Antagonism) and S (synergy), A= ampicillin, AA= ampicillin-acetone extract, AW= ampicillin-water extract, AM= ampicillin-methanol extract, AS= ampicillin-silver nanoparticles, ASA= ampicillin-silver nanoparticles- acetone extract, ASW= ampicillin-silver nanoparticles-water extract, ASM= ampicillin-silver nanoparticles-methanol extract.

7.4.6.1 Fractional inhibition concentration index of conjugated penicillin

Fractional inhibition concentration index of conjugate penicillin was calculated from obtained MIC results of conjugated penicillin and recorded in **Table 7.5**. *E. coli, K. pneumonia* and *S. aureus* were used to the determine mutual effect of penicillin conjugated with plant extracts and nanoparticles. 2 synergies (9.5%) were observed, 8 (38.1%) were additive and 11 (52.4%) were antagonism

Table 7.5: Effect of conjugating penicillin with plant extracts and silver nanoparticles.

Sample	*E. coli*	*K. pneumonia*	*S. aureus*
PA	1 (A)	0.75(A)	1(A)
PM	0.625(A)	1.5(A)	1.45(A)
PW	1.5(A)	63.99(N)	63.99(N)
PS	0.56(A)	17(N)	**0.265(S)**
PSA	5(N)	9(N)	9(N)
PSW	6.5(N)	8.43(N)	144(N)
PSM	**0.4(S)**	19(N)	18(N)

Key: A (Additive), N (Antagonism) and S (synergy), P= penicillin-methanol extract, PA= penicillin-acetone extract, PW= penicillin-water extract, PS= penicillin-silver nanoparticles, PSA= penicillin-silver nanoparticles-acetone extract, PSW= penicillin-silver nanoparticles- water extract, PSM= penicillin-silver nanoparticles-methanol extract, S= silver nanoparticles, A= acetone extract, M= methanol extract and W= water extract.

7.4.6. Fractional inhibition concentration index of conjugated vancomycin

Determination of the mutual influence of vancomycin conjugated silver nanoparticles and plant extracts were done using fractional inhibition concentration index (FICI) with results obtained from the MIC of conjugated vancomycin. The FICI results of conjugated vancomycin are recorded in **Table 7.6**. A total of seven sample were tested against three ATCC bacterial strains. Only 1 synergy (4.8%) was observed, 9 (42.8%) were additive and 11 (52.4%) were antagonism.

Table 7.6: Effect of conjugating vancomycin with plant extracts and silver nanoparticles.

Sample	*E. coli*	*K. pneumonia*	*S. aureus*
VA	2(A)	1(A)	1(A)
VM	5(N)	2(A)	2.29(A)
VW	1.5(A)	64(N)	127(N)
VS	2.25(A)	1.125(A)	2.125(A)
VSA	5(N)	9.5(N)	9(N)
VSW	**0.203(S)**	10(N)	9.15(N)
VSM	11(N)	72(N)	71.5(N)

Key: A (Additive), N (Antagonism) and S (synergy), V= vancomycin, VA= vancomycin-acetone extract, VW= vancomycin-water extract, VM= vancomycin-methanol extract, VS= vancomycin-silver nanoparticles, VSA= vancomycin-silver nanoparticles-acetone extract, VSW= vancomycin-silver nanoparticles-water extract, VSM= vancomycin-silver nanoparticles-methanol extract.

7.5 DISCUSSION

The presented work has demonstrated antibacterial activity of antibiotics conjugated to plant extracts and silver nanoparticles. Ampicillin, penicillin and vancomycin are now less effective on their own against pathogenic bacteria. Bio-conjugation allows the formation of chemical bonds between the biological molecule and stabilizer molecules which is attached to the surface of the gold nanoparticles (Sperling *et al*., 2008). Biomolecules present in the plant extracts (e.g., flavonoids, phenols, peptides, etc.) are the ones that serve as reducing and stabilizing/capping agents for metal ions and can also synthesize nanoparticles (Yazdi *et al*., 2018).

Formation of the new functional groups was identified by FTIR. When ampicillin is conjugated with plant extracts, 1383 (C-H bending) and 1234 (C-O Stretching) observed with acetone extract, and 2953 (C-H stretching) and 1111 (C-O Stretching) were observed with methanol extracts. This functional group is present in the molecule of ampicillin and may be responsible for binding to the

surface of the synthesized silver nanoparticles (Rogowska et al., 2017). The sorption of the antibiotic on the surface of the nanoparticles was evidenced by the appearance of bands characteristic, in penicillin methanol extracts 2988 and 2839 (C-H stretching) were observed as new functional group while 2831 (C-H Stretching), 1401 (C=C isolated) and 1009 (C-O Stretching) bands were observed in vancomycin-methanol extracts.

From well diffusion assay, conjugated ampicillin were effective against all tested bacteria. Though, most effective antibacterial activity was observed when ampicillin conjugated silver nanoparticle and acetone extracts (ASA) against *K. pneumonia*. Simillarly, another study reported a significant increase in zones of inhibition of silver nanoparticles conjugated with ampicillin as compared to ampicillin alone (Naqvi *et al*., 2013). Penicillin showed a massive increase of antibacterial activity when conjugated with acetone extract and silver nanoparticle (PSA) against *E. coli* and *S. aureus* and when vancomycin is conjugated with silver nanoparticles and acetone extracts also show high efficacy as antibacterial agents. Hence, these results indicate that *P. grandiflora* tubers acetone extracts and silver nanoparticles in conjugation with selected antibiotics in this study exhibit effective antibacterial activity.

The minimum inhibition concentration of conjugated antibiotics with *P. grandiflora* extracts and nanoparticles were determined using the micro-dilution method. Minimum inhibition concentration in this study range from 0.8-0.0063 which is comparatively higher values than the results obtained in the findings of Ansari *et al*. (2015). When ampicillin is conjugated with silver nanoparticles and water extracts (ASW) inhibited the growth of *K. pneumonia* giving the lowest MIC value of 0.0063 mg/ml. Penicillin conjugated silver nanoparticles (PS) inhibit the growth of *S. aureus* with lowest MIC value of 0.0125 mg/ml. Polymeric nanoparticles were also reported to display more effective antimicrobial activity against methicillin-resistant *Staphylococcus aureus* in comparison with general antibiotics (Turos *et al*., 2007). The lowest concentration to inhibit the growth of *E. coli* amongst the conjugates was observed when vancomycin was conjugated with silver

nanoparticles and methanol extracts (VSM) with MIC value of 0.0125 mg/ml. This suggests that these nanoparticle conjugates can be used to treat multidrug resistant bacteria according to the study conducted by Kora et al., (2013).

At present, there is a lot of scope and importance for the development of new antimicrobials in the treatment of microbial infections. The latest trend shows that the plant-based antimicrobial agents have an enormous therapeutic potential since they do not show any major side effects on human beings (Poojary *et al*., 2015). Mutual influence of antibiotics, silver nanoparticles and plant extracts in conjugates were determined with fractional inhibition concentration index (FICI). Only one synergy (4.8%) was observed, (42.8%) were additive and (52.4%) were antagonism were observed similarly with conjugated ampicillin and conjugated vancomycin and conjugated exhibited 2 synergies (9.5%), (38.1%) additive, and (52.4%) were antagonism. A study on ampicillin conjugated silver nanoparticles functionalized with polyvinyl pyrrolidone showed maximum antibacterial activity compared to other silver nanoparticles conjugates (Kuete, 2010). This study exhibited significant results compared to the antibiotics alone. Improvements in this regard can be achieved with rapid testing first, and then deciding whether an antibiotic will help the patient or not (Nicolaou and Rigol, 2018).

7.6 CONCLUSION

The overall results indicate that conjugated antibiotics with *P. gandiflora* and silver nanoparticles are medicinally important and can be used to improve the activity of existing antibiotics that have become less effective on their own. There was an increase in the zones of inhibition when the ampicillin, penicillin, and vancomycin were conjugated with *P. grandiflora* tubers extracts and silver nanoparticles. Therefore, they can be used to treat infections caused by multi-drug resistant bacteria if they are non-toxic to humans.

7.7 *REFERENCES*

Abhishek, B., and Hemlata, C., (2014). Antibacterial activity of silver nanoparticles conjugated with antibiotics. *Bionano Frontier* 7:32–35

Ansari, M.A., Khan, H.M., Khan, A.A., Cameotra, S.S. and Alzohairy, M.A., (2015). Anti-biofilm efficacy of silver nanoparticles against MRSA and MRSE isolated from wounds in a tertiary care hospital*. Indian journal of medical microbiology*, 33(1), pp.101.

Barabadi, H., Honary, S., Mohammadi, M.A., Ahmadpour, E., Rahimi, M.T., Alizadeh, A., Naghibi, F. and Saravanan, M., (2017). Green chemical synthesis of gold nanoparticles by using *Penicillium aculeatum* and their scolicidal activity against hydatid cyst protoscolices of *Echinococcus granulosus*. *Environmental Science and Pollution Research*, 24(6), pp.5800-5810.

Brown, A.N., Smith, K., Samuels, T.A., Lu, J., Obare, S.O. and Scott, M.E. (2012). Nanoparticles Functionalized with Ampicillin Destroy Multiple-Antibiotic-Resistant Isolates of *Pseudomonas aeruginosa* and *Enterobacter aerogenes* and Methicillin-Resistant *Staphylococcus aureus*. *Applied and environmental microbiology*, 78, 2768-2774

Dzotam, J.K., Simo, I.K., Bitchagno, G., Celik, I., Sandjo, L.P., Tane, P. and Kuete, V., (2018). In vitro antibacterial and antibiotic modifying activity of crude extract, fractions and 3′, 4′, 7-trihydroxyflavone from Myristica fragrans Houtt against MDR Gram-negative enteric bacteria. *BMC complementary and alternative medicine*, 18(1), p.15.

Kora, A.J. and Rastogi, L., (2013). Enhancement of antibacterial activity of capped silver nanoparticles in combination with antibiotics, on model gram-negative and gram-positive bacteria. *Bioinorganic chemistry and applications*.125, pp. 2156-2167.

Kuete, V., (2010). Potential of Cameroonian plants and derived products against microbial infections: a review. *Planta Medical*, 76(14), pp.1479-1491.

Naqvi, S.Z.H., Kiran, U., Ali, M.I., Jamal, A., Hameed, A., Ahmed, S. and Ali, N., (2013). Combined efficacy of biologically synthesized silver nanoparticles and different antibiotics against multidrug-resistant bacteria. *International journal of nanomedicine*, 8, p.3187.

Nicolaou, K.C. and Rigol, S., (2018). A brief history of antibiotics and select advances in their synthesis. *The Journal of antibiotics*, 71(2), p.153.

Pan, X.S., Yague, G. and Fisher, L.M., (2001). Quinolone resistance mutations in *Streptococcus pneumoniae* GyrA and ParC proteins: mechanistic insights into quinolone action from enzymatic analysis, intracellular levels, and phenotypes of wild-type and mutant proteins. *Antimicrobial agents and chemotherapy*, 45(11), pp.3140-3147.

Poojary, M.M., Vishnumurthy, K.A. and Adhikari, A.V., (2015). Extraction, characterization and biological studies of phytochemicals from *Mammea suriga*. *Journal of Pharmaceutical Analysis*, 5(3), pp.182-189.

Ramkumar, V.S., Pugazhendhi, A., Gopalakrishnan, K., Sivagurunathan, P., Saratale, G.D., Dung, T.N.B. and Kannapiran, E., (2017). Biofabrication and characterization of silver nanoparticles using aqueous extract of seaweed *Enteromorpha compressa* and its biomedical properties. *Biotechnology reports*, 14, pp.1-7.

Rogowska, A., Rafińska, K., Pomastowski, P., Walczak, J., Railean-Plugaru, V., Buszewska-Forajta, M. and Buszewski, B., (2017). Silver nanoparticles functionalized with ampicillin. Electrophoresis.

Samie, A., Obi, C.L., Bessong, P.O. and Namrita, L., (2005). Activity profiles of fourteen selected medicinal plants from Rural Venda communities in South Africa against fifteen clinical bacterial species. African journal of Biotechnology, 4(12).

Saratale, G.D., Saratale, R.G., Benelli, G., Kumar, G., Pugazhendhi, A., Kim, D.S. and Shin, H.S., (2017). Anti-diabetic potential of silver nanoparticles synthesized with *Argyreia nervosa* leaf extract high synergistic antibacterial activity with standard antibiotics against foodborne bacteria. *Journal of Cluster Science*, 28(3), pp.1709-1727.

Saravanan, M., Barik, S.K., MubarakAli, D., Prakash, P. and Pugazhendhi, A., (2018). Synthesis of silver nanoparticles from *Bacillus brevis* (NCIM 2533) and their antibacterial activity against pathogenic bacteria. *Microbial pathogenesis*, 116, pp.221-226.

Sieradzki, K., Roberts, R.B., Haber, S.W. and Tomasz, A., (1999). The development of vancomycin resistance in a patient with methicillin-resistant *Staphylococcus aureus* infection. *New England Journal of Medicine*, 340(7), pp.517-523.

Subbaiya, R., Saravanan, M., Priya, A.R., Shankar, K.R., Selvam, M., Ovais, M., Balajee, R. and Barabadi, H., (2017). Biomimetic synthesis of silver nanoparticles from *Streptomyces atrovirens* and their potential anticancer activity against human breast cancer cells. *IET nanobiotechnology*, 11(8), pp.965-972

Tom, R.T., Suryanarayanan, V., Reddy, P.G., Baskaran, S. and Pradeep, T., (2004). Ciprofloxacin-protected gold nanoparticles. Langmuir, 20(5), pp.1909-1914

Turos, E., Reddy, G.S.K., Greenhalgh, K., Ramaraju, P., Abeylath, S.C., Jang, S., Dickey, S. and Lim, D.V., (2007). Penicillin-bound polyacrylate nanoparticles: restoring the activity of *β*-lactam antibiotics against MRSA. *Bioorganic & medicinal chemistry letters*, 17(12), pp.3468-3472.

Wang, H., Chen, Y., Li, X.Y. and Liu, Y., (2007). Synthesis of oligo (ethylenediamino)-β-cyclodextrin modified gold nanoparticle as a DNA concentrator. *Molecular pharmaceutics*, 4(2), pp.189-198.

Yazdi, M.E.T., Khara, J., Sadeghnia, H.R., Bahabadi, S.E. and Darroudi, M., (2018). Biosynthesis, characterization, and antibacterial activity of silver nanoparticles using *Rheum turkestanicum* shoots extract. *Research on Chemical Intermediates*, 44(2), pp.1325-1334.

CHAPTER EIGHT: GENERAL DISCUSSION AND CONCLUSION

8.1 GENERAL DISCUSSION

Many morden medicines are synthesized from traditionally used medicinal plants and their derivatives contribute more than 50% of total drugs used around the world. In South Africa many traditional medicinal plants have been extensively used for many centuries, yet, some are still used as home remedies. The present research work was carried out with *Pyrenacantha grandiflora* tubers which is a medicinal plant used in the Venda region of Limpopo, South Africa for the treatment of gastrointestinal infection, diarrhea and tooth pain. In chapter 3, different solvents were used for extraction of phytochemicals that are responsible for healing purposes using different solvents. Non-polar and polar solvent were used during extraction, this included boiled and cold water which are the most known and understandable methods used by traditional healers. Other solvent used include chloroform, methanol, ethyl acetate, acetone and dichloromethane extract. However methanol yielded a large amount of extract compared to other solvents.

In chapter 4, phytochemicals from *Pyrenacantha grandiflora* tubers were analyzed using qualitative and quantitative methods. Qualitative methods reveal the presence of phenolics, saponins, alkaloids, tannin, Steroids, terpenoids and flavonoids. The quantitative tests revealed that total flavonoids content are more than phenolic total content. This is the very first study to report on phytochemicals studies on *P. grandiflora* in respect of the part tested. Some phytochemical studies of genus *Pyrenacantha* were done on *Pyrenacantha staudtii* leaves (Falodun *et al*., 2005). These biomolecules may be isolated from different parts of the same plant and if the same plant grows in a different climatic environment.

The radical scavenging activity and IC_{50} values of *P. grandiflora* tubers extract with water, acetone and methanol used as solvents were successfully determined (chapter 4). Antioxidants compounds attack free radicals within the human body and protect them from various diseases (Umamaheswari and Chatterjee, 2008). In this study diphenyl-1- picrylhydrazyl radical (DPPH) was used to evaluate scavenging activity. *Pyrenacantha grandiflora* tuber extracts donation electron resulting in a different color with a large decrease in absorbance of reaction mixture indicating good antioxidant activity (Nunes *et al*., 2012; Krishnaiah *et al*., 2011).

It is possible that this antioxidant activity may be due to the high content of flavonoids. Studies have reported that flavonoids are highly effective scavengers of most oxidizing molecules. Moreover, their derivatives have a wide range of antibacterial, antiviral, anti-inflammatory, anticancer, and anti-allergic activities (Umamaheswari and Chatterjee, 2008). Other studies have shown that methanol extract exhibit the highest total phenolics content (Ao *et al*., 2008). However, in our study we found that acetone extracts are the ones with highest total phenolic and flavonoid contents. Therefore this plant can be used in the food industry because they contain compounds that can retard oxidative degradation of lipids and can improve the quality and nutritional value of food (Kähkönen *et al*., 1999).

In this study mixtures of solvents with variable polarity (non-polar, polar and neutral) with different ratio were used for the separation of the compounds from *P.grandiflora* tubers extracts on TLC silica plate (Chapter 4). TLC profiling of all three *P.grandiflora* tubers extracts gives more than five Rf values confirming that these extracts contain different phytochemicals. This variation in R_f values of the phytochemicals provides a very important clue the in understanding their polarity and also helps in the selection of an appropriate solvent system for separation of pure compounds by column chromatography. This information will help in the selection of an appropriate solvent system for further separation of compounds from these *P.grandiflora* tubers extracts.

Bioautography detected the antimicrobial activity of isolated compounds from *P. grandiflora* tubers extracts (chapter 4). The compounds with antimicrobial activity in this study were present in extracts from relatively non-polar solvents. The results demonstrated well-defined inhibition zones against *E. coli* and *K. pneumonia* in BEA developing system which is non-polar. These findings agreed with previously published results (Masoko *et al*., 2005) that the substances responsible for the antimicrobial activity were mainly non-polar in nature. However, CEF system which has intermediate polarity showed antibacterial activity from acetone extract with the R_f values of 0.353, 0.544 and 0.794 against *E. coli*. Similar results have been reported indicating that acetone and methanol fractions of *Punica granatum* and *Delonix regia* have good activity against methicillin-resistant *S. aureus* (Aqil *et al*., 2005).

The results from FTIR shows that all *P. grandiflora* tuber extracts revealed the presence of Hydroxyl group (OH), C-H stretching and C=C carboxyl ranging from 3306-3153, 2923-2894 and 1689-1557 respectively (Chapter 4). However, the OH wagging within 3306 cm-1 was reported to be related to OH of phenolic compounds (Oliveira *et al*., 2016). The band around 1500 cm^{-1} was reported to relate with CH_3, CH_2, flavonoids and aromatic rings, where the vibrations would be the bending (δ) vibration of C-H and the stretching vibration of aromatics (Silva *et al*., 2014).

In this study, chemical methods of synthesizing nanoparticles were used to synthesize citrate capped silver nanoparticles and PEG-coated gold nanoparticles (chapter 5 and 6). AuNPs and AgNPs in this study seemed to be the best candidates as they have inert surface properties which are responsible for their lack of reactivity within biological systems, there is mounting evidence suggesting that they cause DNA damage (Kang *et al*., 2010). Therefore cytotoxicity of these nanoparticles should be determined on various cell lines since the stabilizing or coating molecules on the surface of nanoparticles the play a major role on cytotoxicity testing (Sperling *et al*., 2008; Cui *et al*., 2012; Freese *et al*., 2012; Vijayakumar and Ganesan, 2012). PEG was chosen in this study because of its non-toxicity, water-solubility, and dispersant stabilization.

Silver and gold nanoparticles were also synthesized using the biological method. *Magnetospirillium magnetotacticum* bacteria was used for synthesis. The surfaces of biologically synthesized nanoparticles progressively and selectively adsorb biomolecules when they contact complex biological fluids. Thus, biological nano-particles are more effective due to the attachment of biologically active components on the surface of synthesized nanoparticles from the biological sources. Moreover, biological nanoparticles have been applied in many biomedical contexts, including anticancer and antimicrobial applications.

Various studies involving characterization of AuNPs have shown that these nanoparticles have a distinctive absorption peak within a wavelength range of 518 –-530 nm (Tom *et al*., 2004; Nirmala Grace and Pandian, 2007). However, the formation of nanoparticles is initially confirmed by the color change of the solution during preparation (Huang and Yang, 2004). In this study, silver nanoparticles change from colorless to yellow color at 434 nm (Chapter 5) and for gold nanoparticle change from pale yellow to ruby red at 530 nm (Chapter 6). However, studies have revealed that particles size may be less important than total charge in term of permeation, therefore further studies can be done to determine the total charge of these nanoparticles.

Transmission electron microscopy serves as a further means to establish gold nanoparticles formation, indicating size, distribution, and morphology of the nanoparticles formed. Silver nanoparticles were in cube shape with size ranging from 5-33 nm and gold nanoparticles were spherical in shape with size ranging from 7-16 nm. In order for effective drug delivery to occur in bacterial cells and to ensure that the nanoparticles should ideally be between 10 and 30 nm in size and spherical in shape, as smaller sizes have been shown to have better antibacterial activity (Arshi *et al*., 2011). Hence these nanoparticles can be used to deliver various drugs due to their small size.

Pyrenacantha grandiflora tubers extracts were conjugated with selected antibiotics and nanoparticles. Tom *et al.* (2004) showed that there is a marked colour change in the citrate-

capped AuNP suspension once conjugation to ciprofloxacin occurs. In this scenario there was no significant color change on conjugated *P. grandiflora* tuber extracts. Furthermore, studies have shown that upon attachment to antibiotics and other drugs, AuNPs display a characteristic aggregation phenomenon (Grace and Pandian, 2007).

In order to investigate bond formation between the functional groups present on the surface of the *P.grandiflora* tubers extracts, gold and silver nanoparticles, ampicillin, penicillin, and vancomycin it was necessary to analyze the infra-red (FT-IR) spectrum of the samples. This indicates whether plant extract has bound with nanoparticles or antibiotic and between which functional groups this bond has formed. Previous studies have indicated that neither the keto nor the carboxyl groups present on the antibiotic molecule are involved in binding to the surface of the AuNPs. The second method of attachment is through bio-conjugation, namely, the formation of chemical bonds between the biological molecule and stabilizer molecules attached to the surface of the gold nanoparticles (Sperling *et al.*, 2008). However, bioconjugation reactions, in most cases, have not been optimized, such that attachment of the desired molecule to the nanoparticle surface is guaranteed. Hence optimization is needed in future studies on this noncovalent interaction of plant extracts with antibiotics and nanoparticles.

Gold and silver nanoparticles exhibit higher antimicrobial activity in gram-negative bacteria as these cells are deemed to internalize the nanoparticles (Selvara and Alagar). For instance, the activity of amoxicillin, ampicillin, erythromycin, kanamycin, and chloramphenicol was reported to be improved in the presence of silver nanoparticles (Fayaz *et al*., 2010; Li *et al*., 2005). Likewise, the coating of vancomycin, streptomycin, gentamycin, neomycin and acridine derivative gold nanoparticles has enhanced their antibacterial efficacy (Mitra *et al*., 2014; Gu *et al.*, 2003; Grace *et al*., 2007). Such techniques of conjugating antibiotics with inorganic materials can be very effective due to the capacity of inorganic metal ions to target multiple sites

of a biological system (Pelgrift, 2013). Another study clearly showed that the citrate-capped AuNPs have no inhibitory effect on bacterial growth of any of the strains tested (Abtahi, 2013).

The overall antibacterial activity of synthesized *P.grandiflora* tubers extracts showed conjugates was reported in Chapter 5. Biologically synthesized silver nanoparticles showed to be less effective compared to chemically synthesized silver nanoparticles. Well diffusion assay results clearly indicated that chemically synthesized silver nanoparticles when conjugated with *P. grandiflora* tubers extracts had good bacterial activity against *E. coli*, *K. pneumoniae* and *S. aureus* when compared to biologically synthesized silver nanoparticles conjugates. The lowest MIC was observed with acetone and methanol extracts of *P. grandiflora* tubers mostly against *E. coli* and *S. aureus*.

In chapter 6, biologically synthesized gold nanoparticles showed to be less active when compared to the chemically synthesized gold nanoparticles with well diffusion assay. The overall results of well diffusion assay revealed that biological synthesized gold nanoparticles conjugated with *P.grandiflora* tubers extracts exhibited antibacterial activity against *E. coli* whereas in chemically synthesized gold nanoparticles more antibacterial activity was observed on both MSSA and MRSA. This suggests that the effectiveness of conjugated plant extract depend on the type of conjugate and the strain tested. Minimum inhibitory concentration of conjugated *P. grandiflora* tubers extracts with biologically and chemically synthesized gold nanoparticles was determined using microdilution assay. Our findings showed that the tuber extracts exhibited strong antimicrobial activity with the smallest MIC of 0.0063 mg/ml which was smaller than the concentration of 1 mg/ml previously reported (Vatsos and Rebours, 2015).

Chapter 7 discussed the functionalization of penicillin, ampicillin and vancomycin. These antibiotics are currently less effective against most pathogenic bacteria. This was confirmed in our study, where these antibiotics showed no antibacterial activity on their own against, *E. coli*, *K. pneumoniae*, and MRSA. Ampicillin antibacterial activity was improved when conjugated with

silver nanoparticles against *K. pneumonia* and *E. coli.* Vancomycin showed improved activity when conjugated to silver nanoparticles against *K. pneumonia*. From the MIC results, penicillin was improved by acetone extracts and vancomycin shows to be more effective when conjugated with silver nanoparticles and water extracts. No bactericidal activity was observed with conjugated *P. grandiflora* with antibiotic or silver nanoparticles.

Fractional Inhibition Concentration Index was calculated on the results obtained from MIC results, 8 synergies (26.7%), 12 additive (40%), and 10 antagonisms (33.3%) were observed from both biological and chemical synthesized gold nanoparticles conjugated *P. grandiflora* tubers extracts. Only 2 synergy, 7 additive, and 6 antagonisms were from biological synthesis this means that more synergy was from chemical synthesized gold nanoparticles conjugates.

8.2 RECOMMENDATIONS

Since *Pyenacantha grandiflora* shows to be effective against bacteria and fungi, it will be interesting to look at Immunomodulatory effect, anti-HIV, anti-cancer and anti-inflammatory activity. Studies on the activities of medicinal plants against other organisms such as parasite, protozoans, viruses, and helminths and the determination of their phytochemistry are recommended.

Toxicity of *P. grandiflora* tubers extracts and its conjugates from this study can be determined in-order to understand the dosage needed in human body that will not cause detriment to life.

It is realized that biological synthesis of nanoparticles utilizing microorganisms is a moderate procedure that can take a few hours to a couple of days comparing with chemical methods. Therefore, lessening of time of synthesis will influence this biosynthesis to course significantly more appealing.

Arrangement and size of particles are two imperative issues in the assessment of nanoparticle amalgamation. In this way, compelling control of the size of particles must be broadly explored. By fluctuating parameters like type of microorganism, development medium, combination of synthesis conditions, pH, temperature, and source compound of target nanoparticle it may be conceivable to acquire adequate control of molecule size and mono-scattered.

Since the control of shape in chemical synthesis of nanoparticles is yet a progressing zone of research, natural procedures with the capacity to entirely control molecule morphology would along these lines offer significant preferred standpoint.

A better understanding of the processes of gene transfer in natural environments is crucial in order to assess the risk of antibiotic resistance among ubiquitous agents of diarrhea such as the motile aeromonads

8.3 CONCLUSION

The present study showed that the combination of acetone and water extracts of *P. grandiflora* has very good antioxidant and antibacterial activity. Such results would guide the establishment of some compounds that could be used to research new and more potent antimicrobials and antioxidants of the plant origin. In addition to being effective against bacteria, these compounds could exhibit inhibitory effects against viruses and parasites. Biosynthesis and characterization of silver nanoparticles were successfully carried out in this study. The In-vitro antibacterial activity of silver nanoparticles conjugated with *P. grandiflora* tubers extracts showed potential antibacterial property against multi-drug resistant pathogens such as *Staphylococcus aureus*, *Klebsiella pneumonia* and *Escherichia coli*. Overall, we reported synthesis of *P. grandiflora* tubers extracts conjugated with silver and gold nanoparticles. Nanoparticles were successfully synthesized separately and characterized by UV–Visible, XRD and TEM analyses and then

conjugated to plant extracts. Identification of conjugate were made by FTIR and antibacterial activity were evaluated. The overall results indicate that *P. grandiflora* and conjugates are medicinally important and can be used for future antibacterial activity. We demonstrated that conjugated antibiotics with *P. grandiflora* and silver nanoparticles are medicinally important and can be used to improve the activity of existing antibiotics that have become less effective on their own. There was an increase in the zones of inhibition when ampicillin, penicillin, and vancomycin were conjugated with *P. grandiflora* tuber extracts and silver nanoparticles. All conjugates formed were active against *E. coli*, *K. pneumonia,* and *S. aureus* very few synergistic antibacterial activities were observed. Therefore, they can be used to treat infections caused by multi-drug resistant bacteria if they are non-toxic to humans.

8.4 *REFERENCES*

Ao, C., Li, A., Elzaawely, A.A., Xuan, T.D. and Tawata, S., (2008). Evaluation of antioxidant and antibacterial activities of *Ficus microcarpa* L. fil. extract. *Food control*, 19(10), pp.940-948.

Arshi, N., Ahmed, F., Kumar, S., Anwar, M.S., Lu, J., Koo, B.H. and Lee, C.G., (2011). Microwave assisted synthesis of gold nanoparticles and their antibacterial activity against *Escherichia coli* (*E. coli*). *Current Applied Physics*, 11(1), pp.S360-S363.

Cui, Y., Zhao, Y., Tian, Y., Zhang, W., Lü, X. and Jiang, X., (2012). The molecular mechanism of action of bactericidal gold nanoparticles on *Escherichia coli.* Biomaterials, 33(7), pp.2327-2333.

Falodun, A., Usifoh, C.O. and Nworgu, Z.A., (2005). Phytochemical and active column fractions of *Pyrenacantha staudtii* leaf extracts on isolated rat uterus. *Pakistan journal of pharmaceutical sciences*, 18(4), pp.31-35.

Fayaz, A.M., Balaji, K., Girilal, M., Yadav, R., Kalaichelvan, P.T. and Venketesan, R., (2010). Biogenic synthesis of silver nanoparticles and their synergistic effect with antibiotics: a study against gram-positive and gram-negative bacteria. *Nanomedicine: Nanotechnology, Biology and Medicine*, 6(1), pp.103-109.

Freese, C., Uboldi, C., Gibson, M.I., Unger, R.E., Weksler, B.B., Romero, I.A., Couraud, P.O. and Kirkpatrick, C.J., (2012). Uptake and cytotoxicity of citrate-coated gold nanospheres: Comparative studies on human endothelial and epithelial cells. *Particle and Fibre Toxicology*, 9, 1-11.

Grace, A.N. and Pandian, K., (2007). Antibacterial efficacy of aminoglycosidic antibiotics protected gold nanoparticles—A brief study. Colloids and Surfaces A: Physicochemical and *Engineering Aspects*, 297(1-3), pp.63-70.

Huang, H. and Yang, X., (2004). Synthesis of polysaccharide-stabilized gold and silver nanoparticles: a green method. *Carbohydrate research*, 339(15), pp.2627-2631.

Kähkönen, M.P., Hopia, A.I., Vuorela, H.J., Rauha, J.P., Pihlaja, K., Kujala, T.S. and Heinonen, M., (1999). Antioxidant activity of plant extracts containing phenolic compounds. *Journal of agricultural and food chemistry*, 47(10), pp.3954-3962.

Kang, M., (2010). Measuring social media credibility: A study on a Measure of Blog Credibility. *Institute for Public Relations*, pp.59-68.

Krishnaiah, D., Sarbatly, R. and Nithyanandam, R., (2011). A review of the antioxidant potential of medicinal plant species. *Food and bioproducts processing*, 89(3), pp.217-233.

Nunes, X.P., Silva, F.S., Almeida, J.R.G.D.S., de Lima, J.T., de Araújo Ribeiro, L.A., Júnior, L.J.Q. and Barbosa Filho, J.M., (2012). Biological oxidations and antioxidant activity of natural products. In Phytochemicals as nutraceuticals-Global Approaches to Their Role in Nutrition and Health. *InTech*.

Oliveira, R.N., Mancini, M.C., Oliveira, F.C.S.D., Passos, T.M., Quilty, B., Thiré, R.M.D.S.M. and McGuinness, G.B., (2016). FTIR analysis and quantification of phenols and flavonoids of five commercially available plants extracts used in wound healing. Matéria (Rio de Janeiro), 21(3), pp.767-779.

Silva, A.J., Silva, J.R., de Souza, N.C. and Souto, P.C., (2014). Membranes from latex with propolis for biomedical applications. *Materials Letters*, 116, pp.235-238.

Sperling, R.A., Gil, P.R., Zhang, F., Zanella, M. & Parak, W.J. (2008). Biological applications of gold nanoparticles. *Chemical Society Reviews*, 37, 1896-1908.

Tom, R.T., Suryanarayanan, V., Reddy, P.G., Baskaran, S. and Pradeep, T., (2004). Ciprofloxacin-protected gold nanoparticles. *Langmuir*, 20(5), pp.1909-1914.

Umamaheswari, M. and Chatterjee, T.K., (2008). In vitro antioxidant activities of the fractions of Coccinia grandis L. leaf extract. African Journal of Traditional, *Complementary and Alternative Medicines*, 5(1), pp.61-73.

Vijayakumar, S. and Ganesan, S., (2012). In vitro cytotoxicity assay on gold nanoparticles with different stabilizing agents. *Journal of Nanomaterials*, pp.14.