Search for new antibacterial compounds by *in vitro* biotransformation of selected antibiotics on market using *pleurotus ostreatus* mushroom

Raphael Matinde (Bpharm)

Masters of Pharmacy in Medicinal Chemistry Dissertation
Muhimbili University of Health and Allied Sciences
November, 2020

Muhimbili University of Health and Allied Sciences
Department of Medicinal Chemistry

SEARCH FOR NEW ANTIBACTERIAL COMPOUNDS BY *IN VITRO* BIOTRANSFORMATION OF SELECTED ANTIBIOTICS ON MARKET USING *PLEUROTUS OSTREATUS* MUSHROOM

By

Raphael Matinde (Bpharm)

A Dissertation Submitted in Partial Fulfilment of the Requirements for the Degree of Masters of Pharmacy in Medicinal Chemistry of

Muhimbili University of Health and Allied Sciences

November, 2020

CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance of a dissertation entitled; **Search for new antibacterial compounds by *in vitro* biotransformation of selected antibiotics on market using *Pleurotusostreatus* mushroom** in fulfilment of the requirements for the degree of Masters of Medicinal Chemistry of Muhimbili University of Health and Allied Sciences.

_____

Dr. Joseph Sempombe

(Supervisor)

Date_____

_____

Prof. Veronica Mugoyela

(Supervisor)

Date_____

DECLARATION AND COPYRIGHT

I, **Raphael Matinde**, declare that this **dissertation** is my own original work and that it has not been presented and will not be presented to any other University for a similar or any other degree award.

Signature……. **Date……**

ACKNOWLEDGEMENT

First and foremost, I express my thanks to the Almighty God for granting me life and good health which indeed facilitated me accomplish this piece of work. I would also like to deliver my gratitude to my beloved mom and siblings for their indefatigable support, hopes and encouragement they gave all the way during my endeavors to successfully complete this work

It is my pleasure to express my exceptional acknowledgement to my sponsor, Catholic University of Health and Allied Sciences (CUHAS) for financial support without which this achievement would just have been a riddle.

I cannot however afford to forget expressing my special thanks to my great supervisors, Prof. Veronica Mugoyela and Dr. Joseph Sempombe for their tremendous and unyielding support and heartily guidance they offered to me during all the time I was struggling to accomplish this task.

Nevertheless, I would like to give my acknowledgements to Muhimbili University of Heath and Allied Sciences (MUHAS) administration especially the Director of Postgraduate studies, Dr. E.Balandya, the Dean of school of Pharmacy, Prof Kennedy Mwambete and the Head of Department of Medicinal Chemistry, Prof. Eliangiringa Kaale for their proper organization to ensure conducive environment which enabled smooth running of this work.

Moreover, I collectively express my special gratitude to the following individuals who spared their precious time to offer me with technical support and experience; Omega Urio, Prof. Sheila Maregesi, Ezekiel Marandu, Amely David Kyando, Ramadhan Adam, Erica Kategere, Raphael Shedafa, George Bwire, Paul Malaba, Boniface Mwita, Edson Ruta, Bertha Mamiro and Alfred Moyo.

Last but not least I would love to say my thanks to all individuals who in one way or another made tremendous contributions to realize this achievement but whose names have not been mentioned above.

DEDICATION

This work is dedicated to my beloved mother, my late father, my young sister, Joyce Isack and my adorable partner N. M. Mashallah. The accomplishment of this task may have not been possible had it not been their love, prayers, hopes, encouragement and unyielding support they gave all time I was endeavoring to accomplish it.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
AMR	Antimicrobial resistance
ARI	Acute Respiratory-tract Infections
CUHAS	Catholic University of Health and Allied Sciences.
DALY	Disability-adjusted life years
ESBL	Extended-Spectrum Beta Lactamases
GARP	Global Antimicrobial Resistance Partnership
HIV	Human Immunodeficiency virus.
INT	Iodonitrotetrazolium
JPEG	Joint Photographic Expert Group
MHA	Mueller-Hinton Agar
MNH	Muhimbili National Hospital
MRSA	Methicillin Resistant *Staphylococcus aureus*
MUHAS	Muhimbili University of Health and Allied Sciences
NP	Normal Phase
PhAC	Pharmaceutically Active Compounds
TB	Tuberculosis
TLC	Thin Layer Chromatography
WRF	White Rot Fungus

LIST OF FIGURES

LIST OF TABLES

DEFINITION OF TERMS

Antibiotic- is achemical substance (medicine) usually obtained from microorganism that is used to prevent the growth bacteriaor treat bacterial infection.

Antimicrobial- is an agent (natural/ synthetic) that kills or stops the growth of microorganisms such bacteria, viruses and fungi. Antimicrobials are grouped according to the microorganisms they act primarily against. For instance, antibiotics are used against bacterial and antifungals are used against fungi.

Antimicrobial resistance- is the ability of a microorganism (like bacteria, viruses, and some parasites) to stop or slow down an antimicrobial (such as antibiotics, antivirals and antimalarials) from working against it. As a result, standard treatments become ineffective, infections persist and may spread to others.

Bioautography- is the planar chromatographic analysis which is hyphenated with the determination of biological activity. It is an effective and inexpensive technique for the phytochemical analysis of plant extracts to identify bioactive lead/scaffolds.

Biotransformation- is a process in which substances (compounds) such as nutrients, amino acids, toxins, and drugs are structurally transformed through enzymatic chemical reactions within a living system. It can also be defined as the series of chemical reactions that occur to a compound, especially a drug, as a result of enzymatic or metabolic activities by a living organism.

Lead compound- is a chemical compound that shows promise as a treatment for a disease and may lead to the development of a new drug. Several compounds are tested in the laboratory to find a lead (“leading”) compound that may act on specific genes or proteins involved in a disease. Once a lead compound has been found, the chemical structure is used as a starting point to make a drug that has the most benefits and the least harms. Finding a lead compound is the first step in making a new drug to treat a certain disease

Standard bacteria- non-clinical isolate bacterial species with known susceptibility profiles used as reference in microbiological studies

ABSTRACT

Background:Research and development of the new antibacterial agents are among the strategized measures to combat antimicrobial resistance(AMR). In the recent decades, biotransformation has been demonstrated as an alternative and effective approach for scavenging the new molecules. There is a high probability of obtaining active metabolites through biotransformation of known compounds possessing antibacterial activities.This study intended to search for new compounds by biotransformation of ceftriaxone and ciprofloxacin using *Pleurotusostreatus* mushroom.

Method: *Pleurotusostreatus* mushroom culture was spiked with antibiotics and incubated for 12 days. The mixture was extracted and analysed by using Thin layer Chromatography (TLC) method to identify newly formed compounds. The TLC chromatograms were then subjected to agar overly bioautography to determine antibacterial activity of the new compounds.

Results: Ceftriaxone and Ciprofloxacin antibiotics were bio transformed to new compounds. The TLC analysis indicated that ceftriaxone was bio transformed to give three new spots and ciprofloxacin gave only one new spot which infer the formation of new compounds from biotransformation by *P. ostreatus* mushroom. Bioautography showed the new spots from biotransformation of ceftriaxone to be active against*S. aureus* both standard and clinical isolates as well as active against MRSA strains. The new spot from biotransformation of ciprofloxacin was only active to standard of E. *coli.*

Conclusion: *P. ostreatus*mushroom has the potential to bio transform antibiotics to form new compounds. This shows biotransformation can be an alternative tool in searching of new compounds. The newly formed compounds from biotransformation showed activity against diseases causing bacteria and hence can be useful in drugs discovery and development.

Key terms; Biotransformation, *Pleurotusostreatus*, Antimicrobial resistance, Bioautography

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Historically, infectious diseases have been a major cause of mortality[1]. However, developments within medicine and public health during the 20th century helped to markedly reduce the burden associated with infectious diseases [2]. The alarming ratcheting up of antimicrobial resistant (AMR) bugs has long been recognized[3]and it has now become increasingly apparent. The increase of AMR has made the management of formerly well and simple managed infectious diseases very difficult to treat. Antimicrobial resistance is one of the most complex global health challenges today.

The world has long ignored warnings that antibiotics and other medicines are losing their effectiveness after decades of overuse and misuse in human medicine, animal health and agriculture. Common illnesses like pneumonia, postoperative infections, diarrheal and sexually transmitted diseases, as well as the world's largest infectious disease killers like tuberculosis are increasingly becoming untreatable because of the emergence and spread of drug resistance[4]. In fact, the AMR has become a significant threat to the health of individuals at the global level. There has been tremendous increase in treatment costs, prolonged hospitalization and increased mortality and morbidity [5]

Reports show that more than 21% of hospital acquired infections are attributable to resistant pathogens [6] and AMR accounts for more than 700,000 deaths every year globally [7]. It is estimated that, Methicillin-resistant *Staphylococcus aureus* (MRSA) kills nearly 50,000 individuals every year in the United States and Europe alone with more dying from it in other parts[8].A team of European researchers estimates that more than 33,000 people in Europe die each year from antibiotic-resistant infections, and that the growing health burden of these infections is similar to that of influenza, tuberculosis (TB), and HIV/AIDS combined [9]. Other antibiotic resistant infections such as TB have had the significant impacts in the

developing countries including Tanzania [10]. It is projected that if this problem (AMR) remains unattended by 2050 it will cost 10 million lives and US$100 trillion every year[7].

In Sub-Sahara Africa, AMR situation is becoming alarmingly terrible. Significant resistance has been reported for diseases such as cholera, dysentery, typhoid, meningitis, gonorrhea and TB [11]. For instance, the reports show that the prevalence of resistance to ampicillin and co-trimoxazole for *Enterobacteriaceae* ranges between 55.6% and 96.7% and between 51.0% and 86.7% respectively while the resistance to chloramphenicol for *Salmonella enterica typhi* ranges between 31.6% and 94.2 for West Africa and between 31.0% and 70.2% for East Africa.The findings also indicate that median prevalence of gentamicin resistance ranged between 16.0% and 35.0% for *E. coli* and 28.6% and 47.0% for *K. pneumoniae* [12].

In Tanzania, despite the limited data, the report from the Global AMR Partnership (GARP) has indicated that resistance of *Streptococcus pneumoniae* to trimethoprim and sulfamethoxazole in children under 5 increased from 25% in 2006 to 80% in 2012, leading to changes in the protocols for treatment acute respiratory tract infections (ARI) in children. *Escherichia coli* from urinary infections showed a 90% resistance to Ampicillin and 30-50% resistance to other common antibiotics. Extended-Spectrum Beta Lactamases (ESBL), which causes resistance to all beta lactam antibiotics, was found in 25-40% of *E. coli* (community and hospital) with more than 50% in children[13].

Four drug-resistant pathogens, namely, third-generation cephalosporin-resistant *E coli,* MRSA, carbapenem-resistant *Pseudomonas aeruginosa,* and third-generation cephalosporin-resistant *Klebsiella pneumoniae*—had the largest impact on health, accounting for 67.9% of the disability-adjusted life years (DALYs) per 100,000 populations globally. Infections caused by colistin-resistant and carbapenem-resistant bacteria accounted for 38.7% of total DALYs [14].

Several measures have however been taken towards the mitigation of this treatment challenge. These include but not limited to implementing antimicrobial stewardship programs, advocacy of rational use of medicines, synthesis and development of new antimicrobial compounds [15].

These measures have demonstrated the low pace in curbing the existence of the AMR**.** Thus, this necessitates the investment of more efforts in searching for new antimicrobial agents.

Synthetic chemistry has always been playing the major role in the scavenging of the new molecules of medicinal importance. There are however challenges associated with this approach including high costs and deleterious impacts on the environment. In recent decades, biotransformation has emerged as an alternative synthetic approach in searching of novel medicinal molecules.

This study aimed at searching for new antibacterial compounds by biotransformation of the standard antibiotics available in the market using *Pleurotusostreotus* mushroom. Many fungi species including *Pleurotusspp* have demonstrated the ability in biotransformation. However, very little is known about utilization of modified molecules by *Pleurotusestreotus* as potential antibacterial compounds/leads for development of bioactive compounds which was the focus of this study.

1.2 Problem Statement

The increase of the AMR pathogens has turned the management of the infectious diseases which were previous easily managed more difficult and costly [16]. There has been tremendous increase in treatment costs, prolonged hospitalization and increased mortality and morbidity due to emergence of the resistant strains [17,18]. It has been reported that resistant bacteria strains are responsible for more than 21% of nosocomial and 700,000 deaths every year globally [7]. In Tanzania despite the limited data, the findings show that, the overall 45.5% bacterial isolate of the most common bacteria in the settings, namely, *E. coli*, *S. aureus* and *Proteus spp* are resistant to at least two antibiotics including Chloramphenicol, Amoxicillin, Gentamicin, Cefuroxime, Ciprofloxacin, Amoxyclav, Doxycycline, Ceftriaxone and Ampicillin which are among the available inexpensive treatment options[19].

Several steps have been taken in response to tackling the AMR problem including but not limited to advocacy of rational use of medicines, synthesis and development of new

antimicrobial compounds. These measures still demonstrate the low pace in curbing the existence of AMR, thus impelling more efforts in searching for new antimicrobial agents.

This study aimed at searching for the new antibacterial compounds by biotransformation of the existing standard antibiotics using the *Pleurotusostreatus* mushroom.

1.3 Conceptual Framework

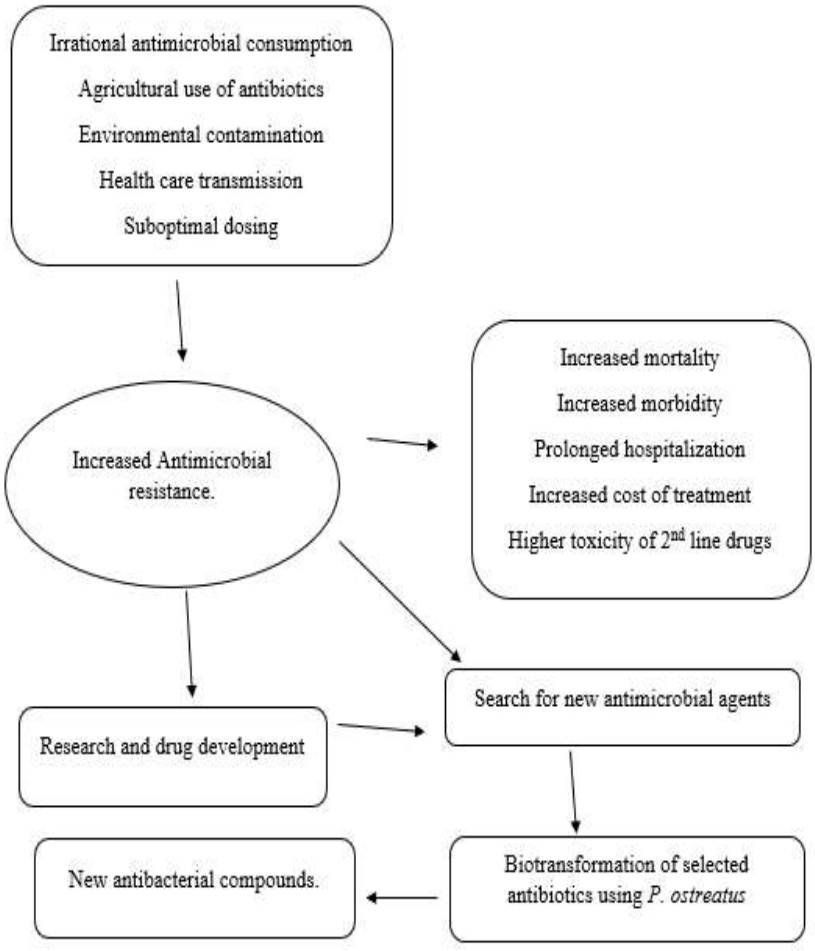


Figure 1.1: The interplay of AMR with associated factors

Narrative of the conceptual Framework

The exaggerated and inappropriate use of antibiotics among many other factors leads to the increase of AMR. Emergency of AMR poses many negative impacts on the community including but not limited to increased mortality and morbidity, prolonged hospitalization and increased treatment costs. The research and development of new antibiotics have been in the forefront in tackling the AMR crisis. Thus, this impels for the searching of new antimicrobial compounds to keep pace with fighting the AMR problem. This study will work on searching for new compounds by biotransformation of selected antibiotics using *Pleurotusostreatus* mushroom.

1.4. Rationale of the study.

This study corroborates efforts to fight against the global disaster of AMR. In the prospect that, this study yields positive results, it will furnish lead compounds with promising antibacterial activity. On optimization of these lead compounds, there might be a possible chance of obtaining new antibacterial molecules which can benefit the community to treat notorious resistant bugs.

On the other hand, this study will demonstrate the biochemical potential of the Pleorotusostreatus mushroom to produce the new compounds which may rather be cumbersome to be produced by the use of conventional synthetic methods. The use of biotransformation means is a simple and environmentally friendly approach.

1.5.Research questions

1. What is the biotransformation potential of*Pleorotusostreatus* mushroom on ceftriaxone and ciprofloxacin to produce new metabolites?
2. Do the metabolites obtained from *Pleorotusostreatus*biotrans formation possess any antibacterial activity against standard bacteria?
3. Do the metabolites obtained from *Pleorotusostreatus*biotrans formation possess any antibacterial activity against resistant bacteria?

1.6.Broad objective

To search for new antimicrobial compounds by biotransformation of selected antibiotics onmarket using *Pleurotusostreatus* mushroom.

1.6.1 Specific objectives

1. To determine by thin layer chromatography method the new metabolites produced after biotransformation of ceftriaxone and ciprofloxacin using *Pleurotusostreatus* mushroom.
2. To evaluate the *in vitro* antibacterial activities of the metabolites produced by *P. ostreatus*against selected standard bacteria.
3. To evaluate the *in vitro* antibacterial activities of the metabolites produced by *P. ostreatus*against the selected antibiotics resistant bacteria.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Biotransformation as an alternative to synthetic approach

Synthetic chemistry has always been playing the major role in the scavenging of the new molecules of medicinal importance. This technique, involves one or more compounds (known as reagents or reactants) that will undergo a transformation when subjected to certain conditions. There are however challenges associated with this approach including high costs and deleterious impacts on the environment.

In recent decades, biotransformation has emerged as an alternative synthetic approach in searching of novel medicinal molecules. It involves series of chemical reactions that occur to a compound as a result of enzymatic or metabolic activities by a living organism. Biotransformation has become an alternative tool with great potential, especially for the development of sustainable technologies for the production of chemicals and drugs, that is, green chemistry[20–23]. This approach has certain advantages over synthetic approach in terms of high stereo- and regioselectivity, fewer side reactions, mild reaction conditions, simple operation procedures, easier separation of products, lower costs and being environmentally friendly[24–27]. Some of the reactions which cannot be achieved by the classical way of organic synthesis are fulfilled by biotransformation approach [28]. A particular compound is modified by transforming functional groups with or without degradation of the carbon skeleton. These modifications result in the formation of novel and useful products that may be difficult or impossible to be obtained through conventional chemical procedures[20,29].

Biotransformation has therefore proved to be a powerful tool in addition to expanding array of organic synthesis for generation of and optimization of the lead compounds in the drug discovery and development [26]. It can be used to carry out specific conversions of the compounds by microorganisms, plants or isolated enzymes as the catalysts[30–32].

Fungal biotransformation has been used to develop lead molecules with the potential to treat various disorders [33].

A number of biotransformation of different compounds has been carried out to produce the modified compounds with improved biological activities. A study was conducted in 2006 by Ji-Hua Liu *et al* in an attempt to develop more potent ant malarial agents. The study involved the biotransformation of artemisinin using the microorganism, *Streptomyces griseus*. From this study, artemisinin **(1)** asstarting substrate was converted into other new compounds, namely; artemisitone-9 **(2),** 9α-hydroxy-artemisinin **(3),** 9β-hydroxy-deoxy artemisinin **(4),** and 3α-hydroxy-deoxyartemesinin**(5)** [34] (Figure 2.1).

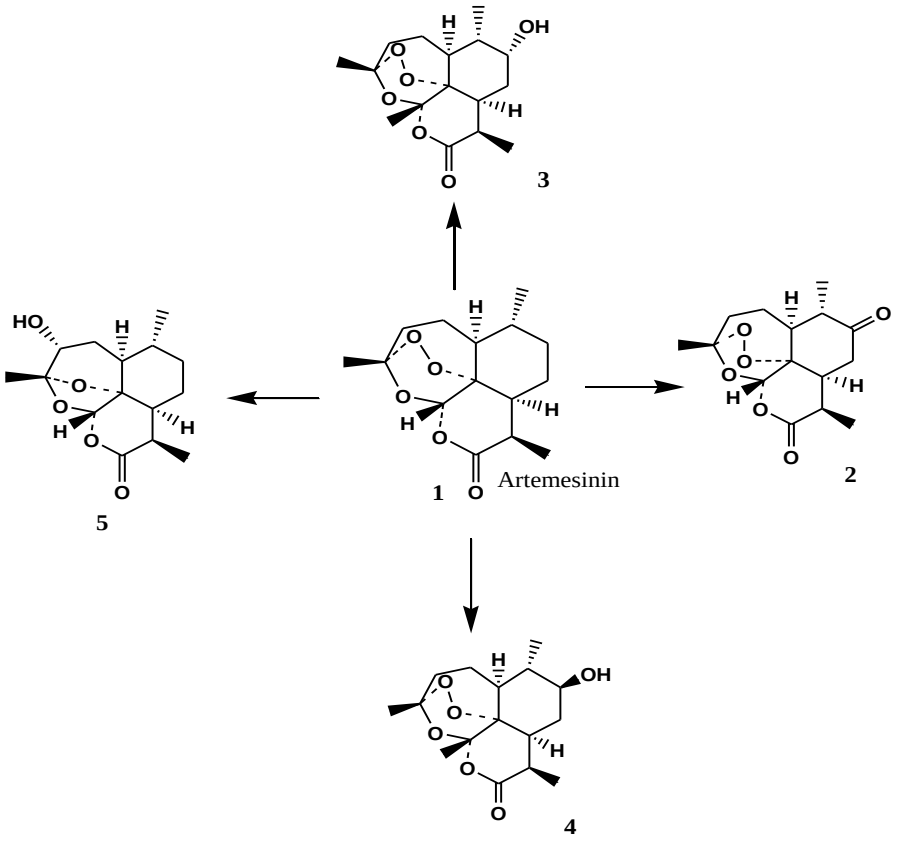


Figure 2.1 Biotransformation of artemisinin by Streptomyces griseus

In another study involving the bioconversion of artemisinin **(1)** using fungi, *Canninghamella echinulate* and *Aspergillusniger* two biotransformed products were identified and structurally recognized as 10β-hydroxy-artemisinin **(6)** and 3α-hydroxy-deoxy artemisinin respectively **(5)** as shown in figure 2.2[35]. This conversion had same biotransformation products as the one observed by the use of *Penicilliumchrysogenum*[36]

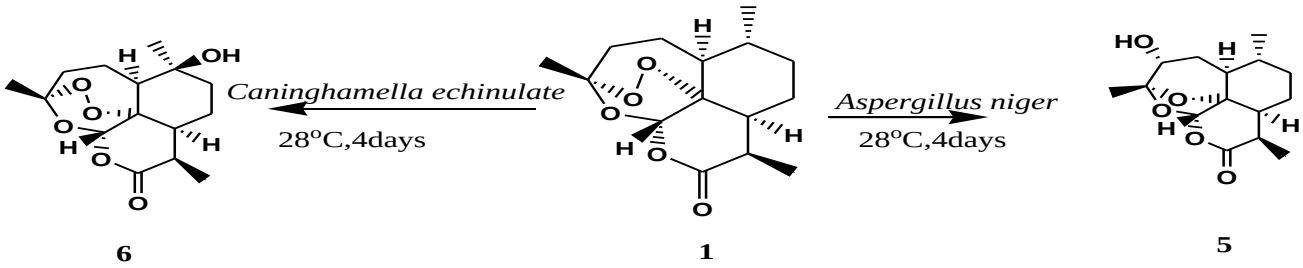


Figure 2.2: Biotransformation of artemisinin by *C. echinulate* and *A. niger*

Many of the fungal metabolites and biotrans formed products have great medicinal importance [37]. Some of the metabolites have been developed as pharmacologically active drugs. For instance, antibiotics, including penicillin G (**7**), cyclosporin (**8**), fusidic acid (**9**) (Figure 2.3) [38].

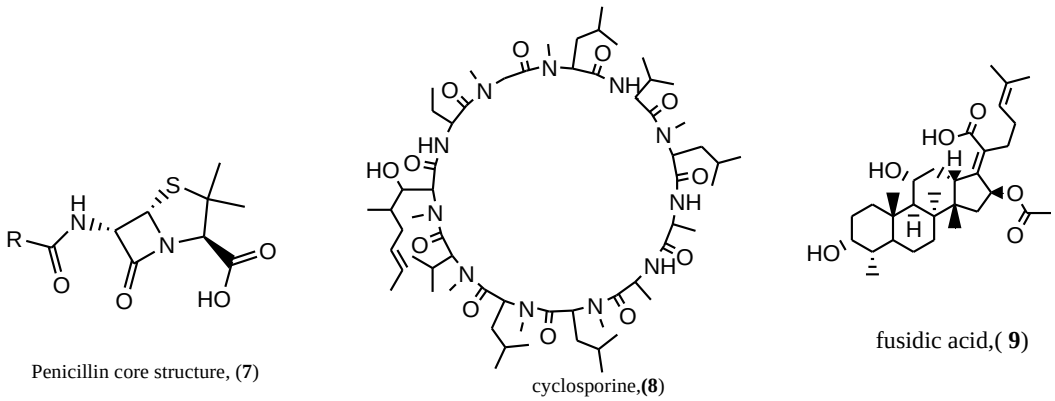


Figure 2.3: Pharmacologically active drugs from biotransformation.

In the study carried out to investigate the effect of the fungus, *Trichotheciumroseum* on the tissue-specific hormone, tibolone **(10)** indicated the bioconversion of the substrate into two new **(14, 15)** compounds and three **(11, 12, 13)** already known compounds as shown in figure 2.4[39].

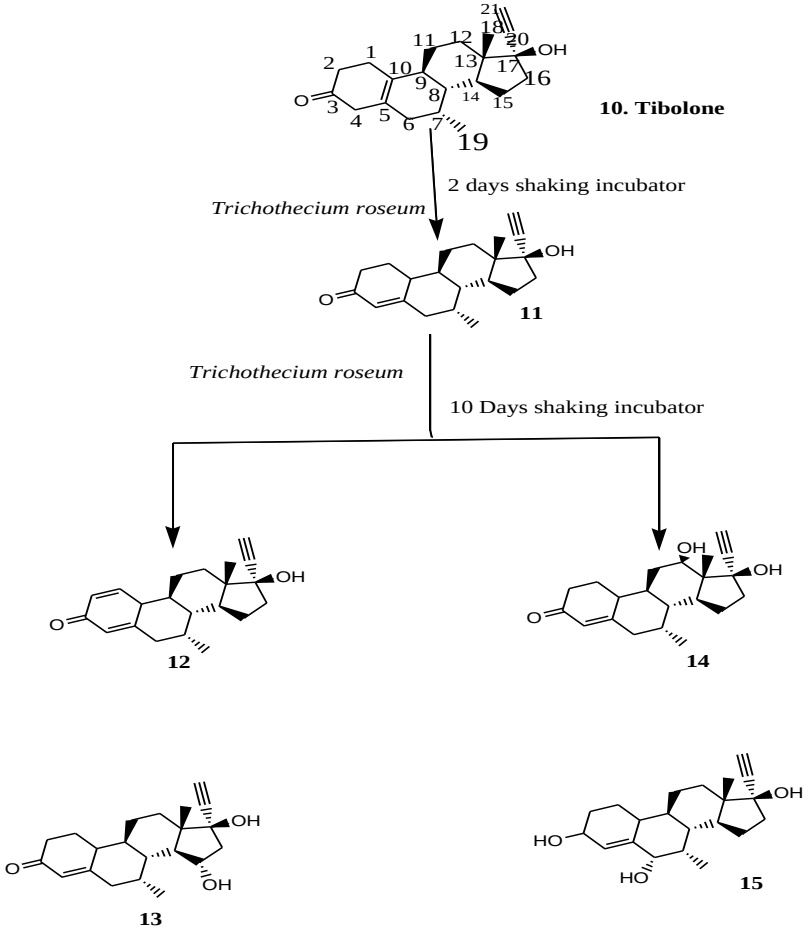


Figure 2.4: Biotransformation of tibolone (1) by a fungal culture *Trichotheciumroseum*

2.2 *Pleurotusostreatus* as a potential catalyst

Pleurotusostreatus is the third most produced edible Mushroom worldwide [40] after *Agaricusbisporus*[41] and *Lentinula edodes*[42]. As members of the white-rot fungi (WRF), *Pleurotusspp* present the ability to grow on a variety of lignocellulosic biomass substrates and degrade both natural and anthropogenic aromatic compounds. This occurs by virtue of the presence of non-specific oxidative enzymatic systems, which consist mainly in laccases, manganese peroxidases (MnPs) and versatile peroxidases (VPs) [43].

White rot fungus (WRF) are physiological group comprising fungi that are capable of biodegrading lignin and the name white rot derives from the white appearance of the wood attacked by WRF, where lignin removal gives a bleached appearance[44]. The study reports have indicated the biochemical capability of the mushroom, *Pleurotusostreatus* to bioconvert lignocellulose[44,45].

*Pleurotusostreatus*has been shown in several studies as the potential WRF for removing the recalcitrant pharmacologically active compound (PhACs) such Carbamazepine and Clarithromycin from the environment (figure 2.5 below). Several mechanisms have been suggested including enzymatic degradation by CYP P450 [46].

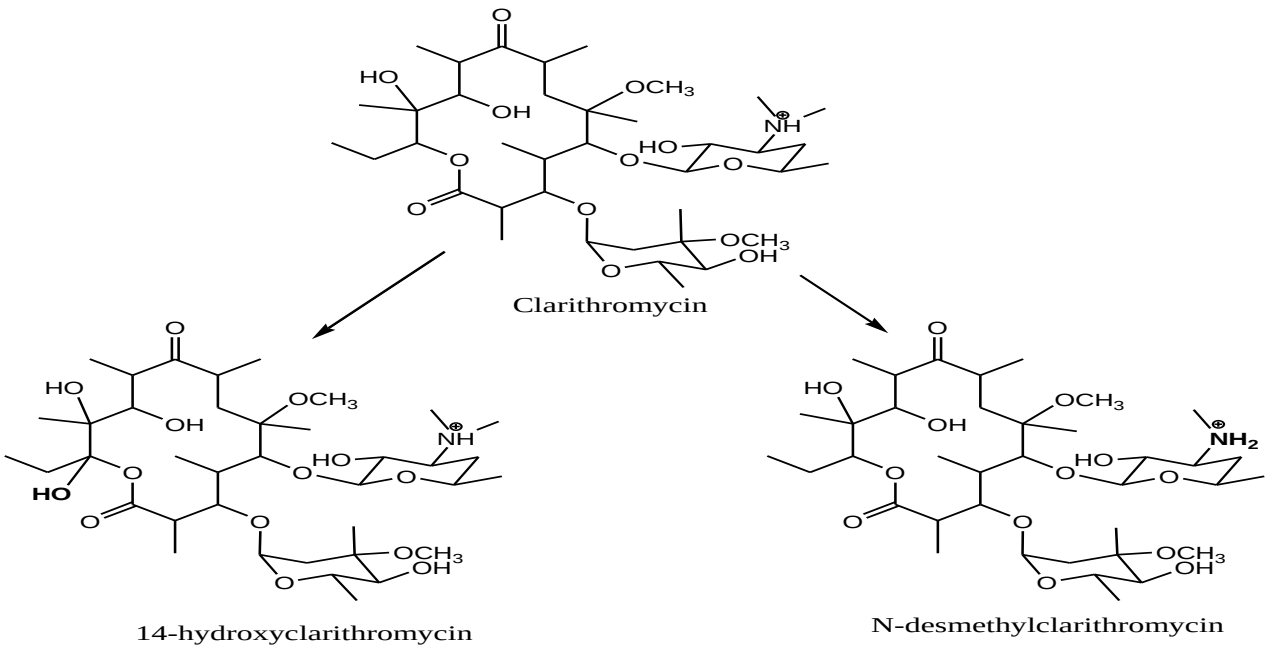


Figure 2.5: Biotransformation pathways of clarithromycin by *P. ostreatus*

Another study conducted by Olicon-Hernandez *et al* shows the role of *Pleurotusostreatus* in biotransformation of the PhAC, ciprofloxacin as indicated in the figure 2.6 below[47].

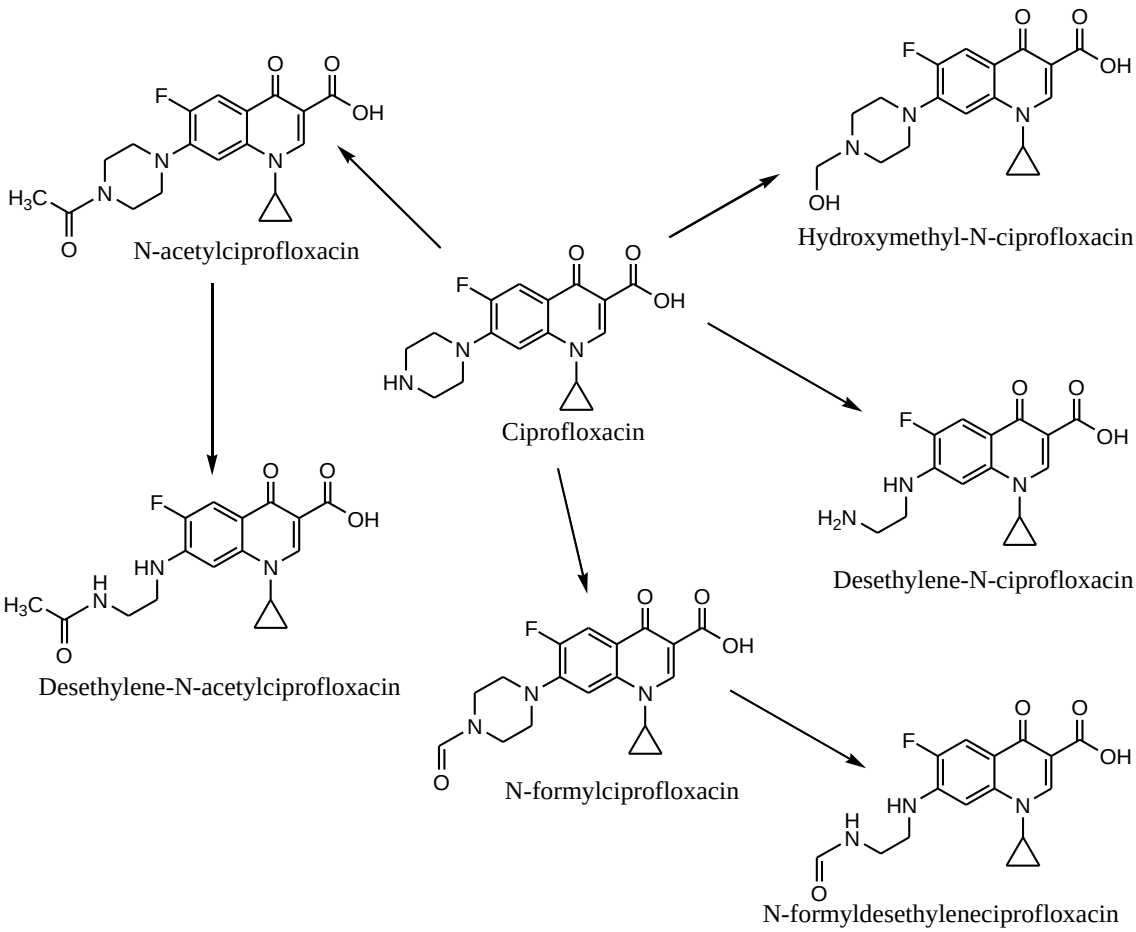


Figure 2.6: Different biotransformation pathways of ciprofloxacin by *P. ostreatus*

Recently, the capability of white rot fungi (WRF) for biodegradation of xenobiotics and recalcitrant pollutants has generated a considerable research in the area of industrial/environmental microbiology.

However, little is still known about the biochemical potential of *Pleurotusostreatus* on the biotransformation of standard antibiotics and possibility of any produced metabolites having antibacterial activities and thus prompted further study in this area.

2.3 Antibacterial activity detection by bioautography

In the effort to discover new lead compounds, there has been the screening of extracts from different sources to detect secondary metabolites with relevant biological activities. With this respects, several bioassays were developed for screening purposes [48]. Among the existing techniques, bioautography is reported to be highly efficacious for assay detection of the antimicrobial compounds due to its capacity to allow activity localization even in the complex matrix, and thus facilitates the target-directed isolation of the active constituents[49–51]. It is also reported to be fast, cheap and permits directed fractionation of the bioactive compounds [52].

Three bioautographic methods namely, agar diffusion or contact bioautography, direct bioautographic detection and immersion or agar overlay are used to detect antimicrobial agents in a mixture of compounds [53]. Agar overlay which was employed in this study, is a combination of contact and direct bioautography. In this method, the separated chromatogram was covered with a seeded molten agar medium. After solidification, incubation and staining with iodonitrotetrazolium(INT) the inhibition or growth bands were visualized[54–56]

CHAPTER THREE

3.0 METHODOLOGY

3.1. Materials and equipment

Pleurotusostreatus mushroom culture slants were obtained from Tanzania Industrial Research and Development Organization (TIRDO). The slants were transported at 8^{o}C from TIRDO to Pharmaceutical microbiology laboratory at MUHAS and stored in the refrigerator at 4°C until the time when they were to be used.

The selected antibiotics on market were purchased from accredited local suppliers. These antibiotics were ceftriaxone (Cefrisoone-1000®, Akums Drugs & Pharmaceuticals Ltd, India) and ciprofloxacin (Ciprox®, Otsuka Pharmeceutical India Private Limited, Ahmedabad, India). They were in pharmaceutical dosage forms, ceftriaxone in powder for injection, 1 g and ciprofloxacin in solution for injection, 2mg/ml. The antibiotics were stored in a cool and dry place following the manufacturers' recommendations until the time to be used.

The mushroom culture media components were purchased from local suppliers. They included peptone water (REF M028-500G, LOT 0000328875, Code No 8902729011270, HiMedia Laboratories Pvt. Ltd, India), dextrose (Emparta®, 1.9425.0521, Merck Life Science Private Limited), magnesium sulphate heptahydrate (Cas No.10034-99-8, Un No. N/R, Tariff:2833 21 00, Batch No. AIPL/974/33, Labtech Chemicals) and potassium dihydrogen phosphate (RPE, Batch No.8B243108C, Code No. 471686, CAS N0. 7778-77-0, Carlo Erba Reagents) all for laboratory use. They were stored in a cool and dry place by observing all manufacturers' recommendations until the time of use.

The analytical grade solvents including methanol (MRS Scientific Ltd), ethanol, ammonia solution (Carlo Erba Reagents), acetone and ethyl acetate (S.D Fine-Chem Ltd) were also purchased from local suppliers.

CamagLinomat 5 version 3.0 sample applicators (Camag, Muttenz, Switzerland) and Camag Visualizer version 4.0 installed in pharmaceutical analysis laboratory were employed.

3.2. Mushroom cultivation.

Mushroom cultivation was performed in the pharmaceutical microbiology laboratory at MUHAS. It was conducted according to the established proceduresby Winska*et al* 2016 [57]. All necessary materials were gathered and put in place. The mushroom culture media was then prepared.

3.2.1. The mushroom culture media preparation

In preparation of media, 20.0 g of dextrose, 15.0 g of peptone water, 5.0 g of potassium dihydrogen phosphate and 2.0 g of magnesium sulphate heptahydrate were weighed on the balanceand put into a 1000 mlErlenmayer flask. Then, 500 ml of distilled water was added and the contents were shaken to completely dissolve. Finally, the solution was made to the mark by distilled water. The same procedure was repeated to prepare 2 L of the media.

The media solution was divided into 400 ml portions in five 1000 ml Erlenmayer flasks. The flasks were top covered with aluminium foil, attached with sterilization indicator and sterilized in an autoclave at121℃ for 30 minutes. After sterilization, the media was then allowed to cool to room temperature.

After cooling, a small amount of *Pleurotusostreatus* mushroom culture from priorly sub-cultured stock on agar was inoculated into the media using a sterile spatula. The flasks were labelled, top covered with aluminium foils and incubated in an incubator at 26℃. The temperature logger device was included to monitor daily temperature. The culture media was intermittently shaken once every day.

3.2.2. Antibiotics incorporation

After 72 hours of incubation, the antibiotics, ceftriaxone and ciprofloxacin, selected based on resistance profile were added into the mushroom culture media. Prior to addition, solutions for antibiotics were prepared. Ceftriaxone powder was dissolved in distilled water to make 20 mg/ml concentration. Ciprofloxacin solution was used at its 2 mg/ml concentration.

Thereafter, 40 ml of each antibiotic solution was added to two separate culture media leaving one media plain as the control. The media flasks were then labelled with type of antibiotic added and re-incubated under the same conditions for further 12 days.

Figure 3.1*: P. ostreaus*mushroom culture at inoculation (a) and at the incubation end (b).

Figure 3.2: *P. ostreatus*mushroom culture as placed in the incubator.

3.3. Crude fungal material extraction

Crude fungal material extraction was done in pharmaceutical analysis laboratory. After 12 days of incubation, the mushroom culture was removed from the incubator and extracted. The liquid media from each flask was shaken and filtered using the What man filter paper (Cat No. 1001 150, What man International Ltd, England). The filtrate was properly collected into plastic container, securely sealed and labelled.

Thereafter, the filtrate was transferred to Institute of Traditional Medicine laboratory, frozen at -80 ℃ and freeze dried. After freeze drying, the gummy material thus obtained from each flask was dissolved in 50 ml of methanol. This solution was then securely stored in a glass container and labelled as aliquot 1.

On the other hand, the hyphaelresidue that remained in the flask was soaked with 50 ml of methanol for 72 hours with intermittent agitation. The mixture was then filtered using What man filter paper (Cat No 1001 150). The filtrate was collected in the glass container labelled as aliquot 2 and no further processing was done on this. The two aliquots were then combined in one container and used as crude extract for analysis. The mixture was safely stored in a refrigerator until the time of use.

3.4. Thin layer Chromatography (TLC) Profiling.

Thin layer chromatography (TLC) profiling was conducted in pharmaceutical analysis laboratory. Analytical process entailed four major steps: sample preparation, sample application plate elution and visualization

3.4.1. Sample Preparation

In each analysis there were three categories of samples, namely, standard, extract and control. The standard was the known antibiotic which was used as positive control, the extract, comprised antibiotic containing media culture crude extract and the plain media culture crude extract served as negative control.

The standard samples were prepared from priorly prepared stock solutions of antibiotics. Ceftriaxone standard sample was prepared by diluting 2 ml of 4 mg /ml aqueous stock solution with methanol to 20 ml. On other hand, ciprofloxacin standard sample was prepared by diluting 2 mg/ml stock solution to 0.05 mg/ml.

The ceftriaxone containing culture crude extract sample was prepared by a two times dilution of the crude extract while the ciprofloxacin containing culture sample, was prepared by a five

times dilution of the crude extract. The control sample was prepared by a two times dilution for the crude extract. The prepared samples were then securely stored in clean well labelled 100 ml round bottomed flasks.

3.4.2. Sample Application

The sample application for all samples was done using the CamagLinomat 5 version 3.0 sample applicator (Camag, Muttenz, Switzerland).Each sample was in turn drawn using Camag syringe (Hamilton, Switzerland) of 100 µl and placed in the syringe slot for introduction on the TLC plate. The application was performed on 10 cm x 10 cm aluminium backed TLC plate coated with analytical grade silica gel 60, normal phase (NP), F_{254}(Lot HX85859754, 1.05554.0001, Merck KGaA, Germany). The origin (application) point was set at 8.0 mm position and solvent front end was set at 80 mm position. The volume of the sample applied for each track was set to be 5 µl and the distance between tracks was automatically determined by the machine. The command for sample application was given using the computer software, WinCATS 4.0 version. After sample application, the plate was allowed to air dry for 20minutes before being subjected to elution.

3.4.3. Plate elution/ Development

After air drying of the plate for 20 minutes, it was followed by plate elution/ development. Plate development was done in the twin trough Camag Tank (Chamber) of 20 cm x 10 cm containing the mobile phase constituted with the mixture of different analytical grade solvents depending on the nature of the samples applied. This step began with preparation of the mobile phase to be used and saturation of the chamber for a period of 20 minutes.

For ciprofloxacin analysis the mobile phase was prepared with the mixture of three solvents constituted as acetone, 30 % ammonia solution and distilled water (28: 5: 5 v/v/v). To prepare the mobile phase, 56 ml of acetone was measured using the measuring cylinder of 100 ml capacity and poured into a clean round bottomed flask of 100 ml capacity. Then, 10 ml of 30 % ammonia solution was again measured using a measuring cylinder of 20 ml capacity and

added into the previously measured acetone in the round bottomed flask. Finally, 10 ml of distilled water was then added to the mixture in the round bottomed flasks. The flask was then stoppered and labelled as ciprofloxacin mobile phase and placed on the works bench.

The mobile phase for ceftriaxone constituted the mixture of five solvents namely, ethyl acetate, acetone, methanol, water and 30 % ammonia solution (5: 2: 2.5: 2.5: 0.5 v/v/v/v/v). Firstly, 20 ml of ethyl acetate was measured by using a 25 ml measuring cylinder and put into a 50 ml flat-bottomed flask. Then, 8 ml of acetone was measure in a 10 ml measuring cylinder and added to the flat-bottomed flask. Then after, 10 ml of methanol and distilled water were separately measured added to the mixture in the flask. Finally, 2 ml of 30 % ammonia solution was measured using a measuring cylinder of 5 ml capacity and topped onto the mixture in the flask. The flask was then stoppered, labelled and placed on the work bench.

After preparation of the mobile phase, the elution chamber (tank) was then saturated for 20 minutes prior to elution process. For saturation and elution, 10 ml of the previously prepared mobile phase was measured using a 20 ml measuring cylinder. This volume was then poured onto the 20 cm x 10 cm filter paper resting on one side of the tank to wet it. The tank was then tilted to transfer the solvent system to another trough of the tank. The tank was then covered with its lid a left for 20 minutes to saturate.

The dried TLC plate was then quickly placed in vertical position on the side containing the mobile phase, opposite to that with the filter paper.

The chamber was closed and mobile phase was let to drift until it reached the development end mark at 8 cm. The plate was removed from the chamber and held in vertical position to allow any excess mobile phase to drip off. The separation was carried out at room temperature. The developed TLC plate was allowed to air dry for five minutes before subjection to visualization.

3.4.4. Visualization

Visualization of the plates was done using Camag Visualizer version 4.0. After derivatization with iodine vapour, the plate was fixed at its slot in the machine and the image of the developed TLC plate was taken under a UV lamp at 254 nm and 360 nm. The image was captured and saved in the form of a Joint Photographic Expert Group (JPEG) file. The image was then resized and cropped at 10 cm x 10 cm, in accordance with the plate dimensions and saved at the resolution of 60 pixels cm^{-1}.

3.5. Bioautography

Bioautography was conducted in pharmaceutical microbiology laboratory. Bioautography was carried out to detect any antibacterial activity of the newly formed molecules from biotransformation of antibiotics. Agar overlay Bioautography technique was employed in this case. Three bacteria, *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli* both standard and clinical isolates were used as indicator organisms.

Bacteria culture was prepared by using Muller Hinton agar (CM0337, OXOID LTD, ENGLAND). 7.6 g of agar was weighed and put into an Erlenmayer flask of 500 ml capacity and 200 ml of distilled water was added to the flask. The flask was shaken to thoroughly dissolve the content. This solution was then autoclaved at the temperature of 121℃ for 30 minutes. After sterilization, the content was contained in the autoclave and the temperature was allowed to fall to 50℃. The media was removed from the autoclave and slowly poured onto the freshly developed and dried chromatographic plates placed in the lids of micro dilution well plates to cover them completely with Muller Hinton agar. The media was allowed to cool and solidify before inoculation of bacteria.

After solidification of the media onto the chromatographic plates, the freshly prepared suspension of bacteria, was slowly applied to the plates by cotton swabs and tilted in all directions to allow the bacteria suspension (0.5 McFarland) to cover all the media. The plates were then overturned to remove any excess suspension liquid. The bacteria cultures on the

plates were properly arranged in the polyethylene box lined with moist cotton wool and placed in the incubator at the temperature of 37℃.

Figure 3.3: Bacteria culture as placed in the incubator.

The bacteria culture on the TLC plates was incubated for 24 hours before visualization to determine antibacterial activities. For visualization of antibacterial activities, the plates were sprayed with 0.2% freshly prepared aqueous solution of Iodonitrotetrazolium (INT). The plates were then re-incubated for 30 minutes and visualized. The zones of inhibition were observed as whitish regions against the purple background

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1. Results.

This study involved the biotransformation of two selected antibiotics on market namely ceftriaxone and ciprofloxacin. The two antibiotics were separately incubated with *Pleurotusostresatus* mushroom culture to study their biotransformation.

4.1.1. Biotransformation results for Ceftriaxone

Chromatographic analysis of extracts indicated one spot on a standard track, six spots on the extract track and two on the control track. The Rf values of these spots were computed and summarized as shown in table 4. 1.The first track from the left was for standard sample (known ceftriaxone) which was used as the positive control. The second track is for mushroom extract mixture exposed to ceftriaxone which was analyzed to determine any biotransformation products. The third track contains the mushroom extract mixture not exposed to ceftriaxone which was regarded as the negative control. The analysis was repeated in the respective manner to the duplicate.

Table 4.1: RF values for ceftriaxone biotransformation product spots.

Track	**Spot distance (mm)**	**Solvent distance (mm)**	**Rf value.**
Standard	20. 0	72. 0	0. 28
	16. 0	72. 0	0. 22
	20. 0	72. 0	0. 28
	24. 0	72. 0	0. 33
Extract	29. 0	72. 0	0. 40
	51. 0	72. 0	0. 71
	55. 0	72. 0	0. 76
	16. 0	72. 0	0. 22
Control	29. 0	72. 0	0. 40

On analyzing the three tracks, there was only one spot band on the extract track which tallied with a spot present on the standard track. This provided inference that the crude extract of ceftriaxone containing culture had some residue of a parent antibiotic. Also, two spot bands on extract track corresponded to the spot on the control track. This provided implication that the spots were for compound from the mushroom itself.

On the other hand, three spots on extract track appeared on neither standard nor control track. They only appeared on the extract track giving inference that these were spots for compounds that resulted from the biotransformation of ceftriaxone by *P. ostreatus* mushroom. This information is summarized in figures 4.1, 4.2 and 4.3 below.

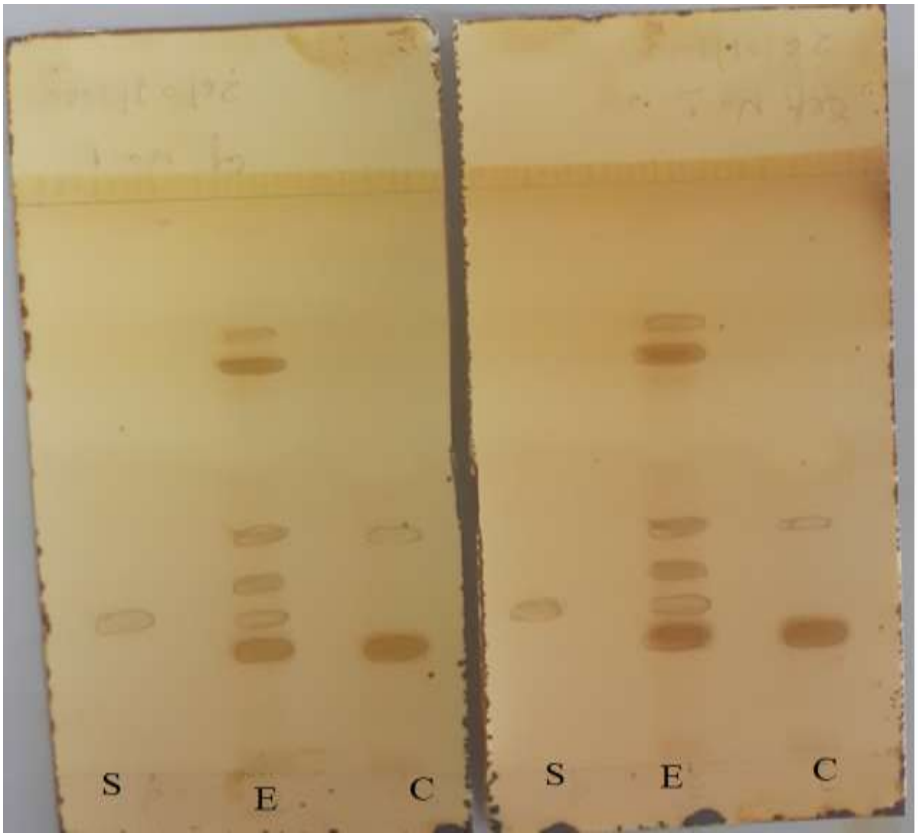


Figure 4.1: Ceftriaxone biotransformation chromatograms image captured by the phone camera as seen invisible light following derivatization with iodine crystals vapor.

S = standard, E = extract, C = control.

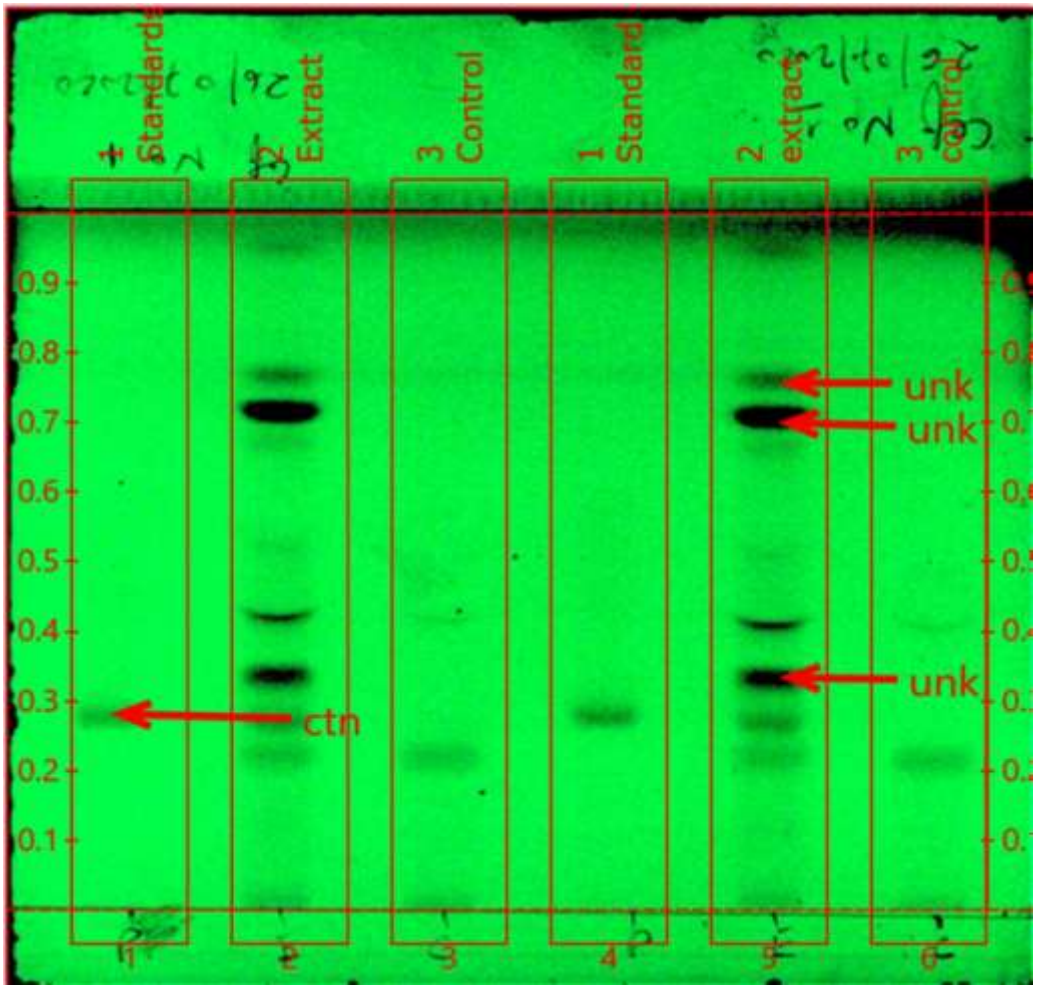


Figure 4.2: Ceftriaxone biotransformation chromatogram image as captured by Camag visualizer camera at 254 nm. Where, Ctn = ceftriaxone, unk = unknown compounds from biotransformation.

When the plate was scanned under Uv-visible the spectrum shown in figure 4.3 below was obtained. The standard tracks are labelled 1, 4, extract track labelled 2, 5 and control track labelled 3, 6.

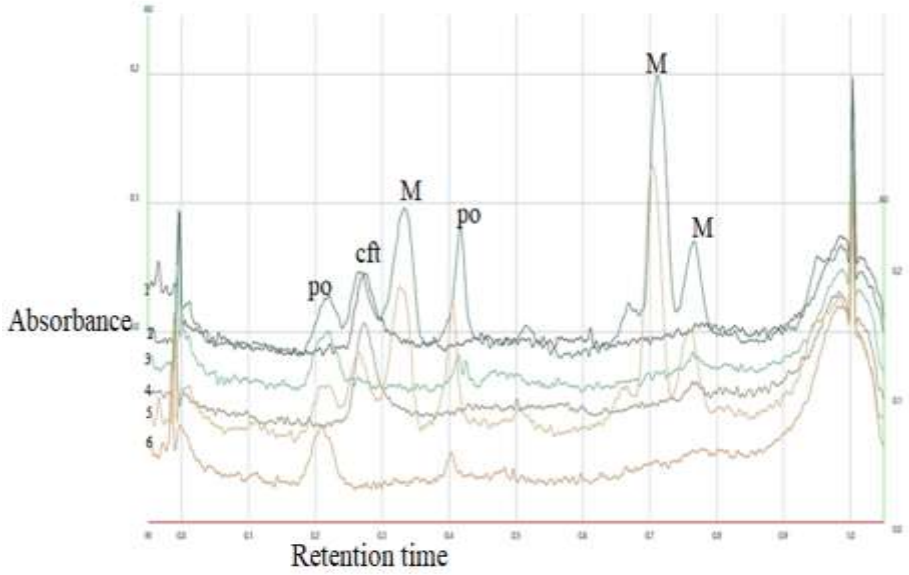


Figure 4.3: Ceftriaxone biotransformation spectrum at 254 nm.

Po = mushroom compound, cft =standard antibiotic, ceftriaxone, M = biotransformation metabolites,

4.1.2. Results for Ciprofloxacin Biotransformation

Ciprofloxacin chromatographic analysis showed one spot on standard track, two on extract and none on the control. The Rf values were calculated and summarized in table 4.2. Sample spotting was done in three tracks, the first representing known antibiotic (ciprofloxacin), the second being for crude extract of *Pleurotusostreatus* mushroom culture which was exposed to ciprofloxacin antibiotic and the last track is for crude extract of *Pleurotusostreatus* mushroom which was not exposed to ciprofloxacin antibiotic.

Table 4.2: Rf values for ciprofloxacin biotransformation product spots.

Track	**Spot distance (mm)**	**Solvent distance (mm)**	**Rf values.**
Standard	28. 0	72. 0	0. 39
	19. 0	72. 0	0. 26
Extract	29. 0	72. 0	0. 4
Control	__	72.	__

On analyzing the three tracks it can be identified that, the first track contains only one spot band which shows the presence of one compound which in this case is a known ciprofloxacin antibiotic. On the hand, the second track of the chromatogram contains two spot band one of which corresponds to the spot on first track, implying the presence of ciprofloxacin residue in the sample of extract. The second spot appears on neither first nor second track. This gives inference that, this second spot must be a new compound resulted from biotransformation of ciprofloxacin by *Pleurotusostreatus* mushroom. This information is summarized in figure 4.4 below.

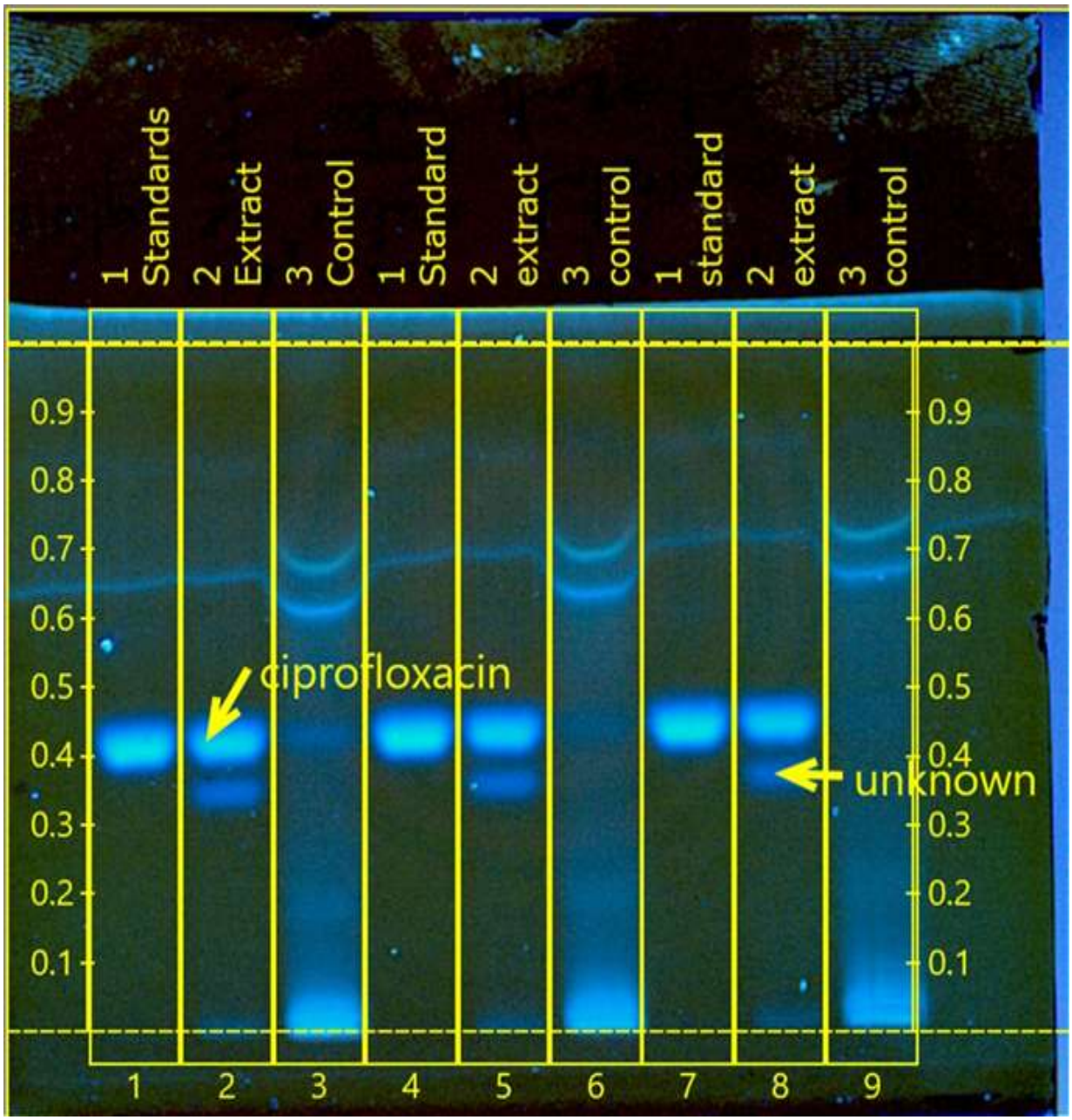


Figure 4.4: Chromatogram of ciprofloxacin biotransformation.

The plate was scanned in the UV-visible at around 360 nm. The spectrum obtain is illustrated in figure 4.5 below. The standard track is shown by numbers 1, 4, 7, extract track by number 2, 5, 8 and control track 3, 6, 9.

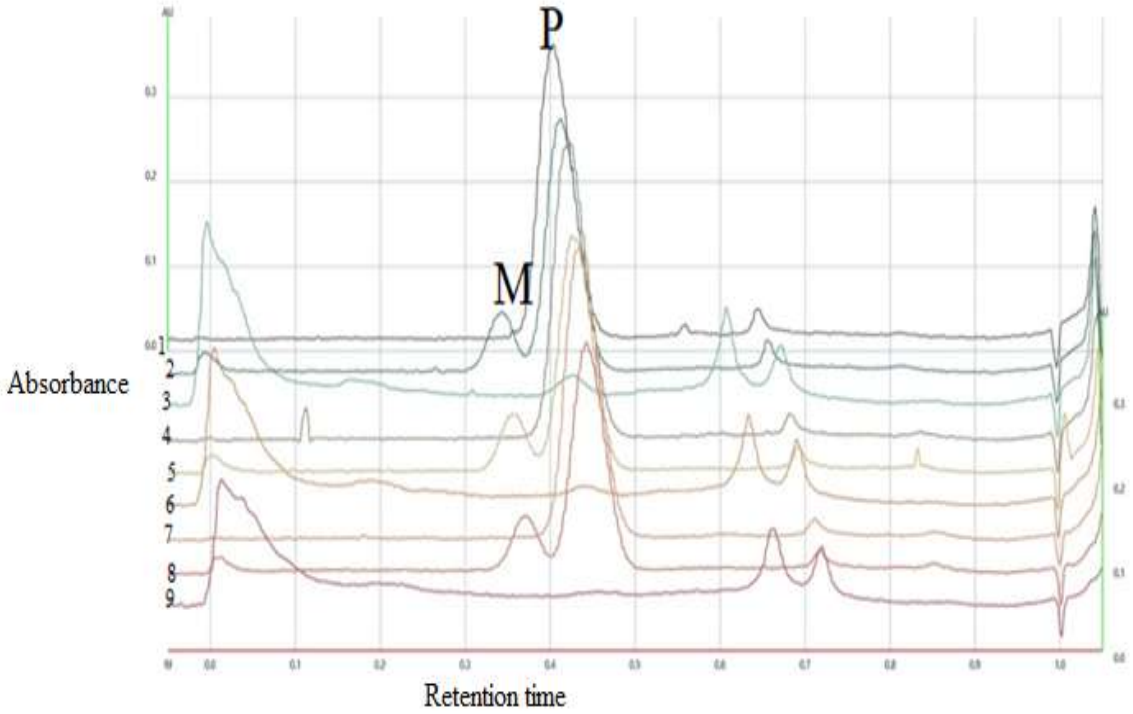


Figure 4.5: The spectrum of ciprofloxacin biotransformation at 254 m

M = metabolites peak, P = parent antibiotic.

4.1.3. Bioautography results

The developed chromatograms were tested for antibacterial activities against three bacteria selected according to Pathogen Priority for Drug Research and Development. These bacteria were *Staphylococcus aureus* representing gram positive bacterial species, *Escherichia coli* representing gram negative bacterial species and *Pseudomonas aeruginosa* selected as one of the most common notorious bugs in our settings.

Upon testing the antibacterial activities, the new spots from biotransformation of ceftriaxone showed the activity against both standard and clinical isolates of *S. aureus* as shown in the bioautogram results in figures 4.6 and 4.7 respectively below. The activities of the new spots were just comparable to that of parent medicine. However, upon testing on the known strain of Methicillin Resistant Staphylococcus aureus (MRSA), only one new spot showed activity although with reduced intensity compare to that on standard and clinical isolates (figure 4.8)

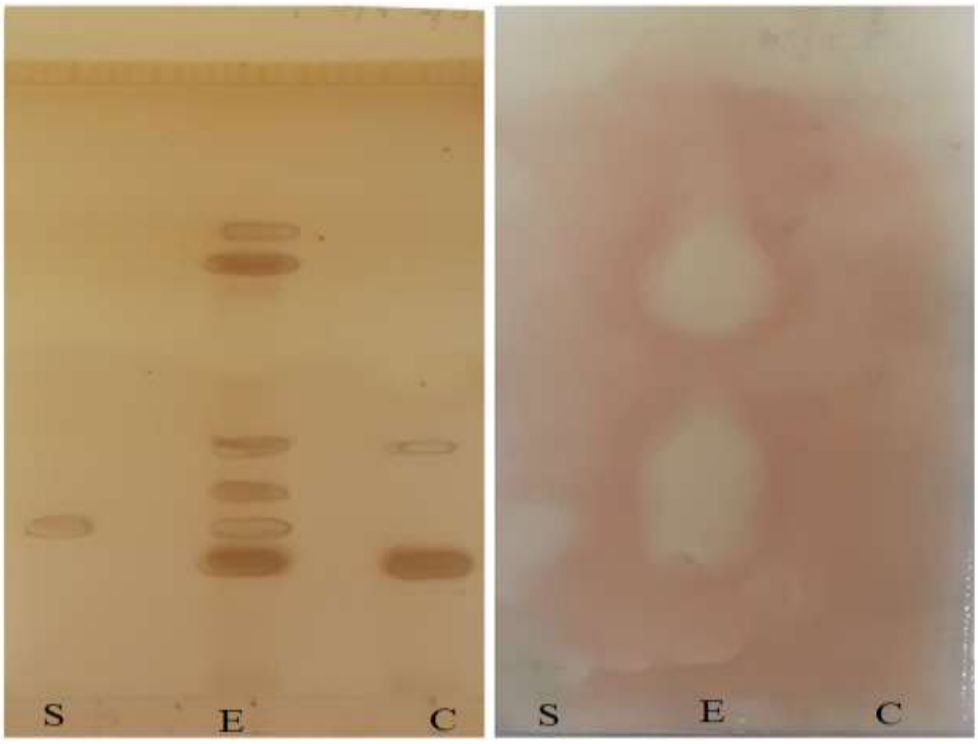


Figure 4.6: Bioautogram(right)of standard *S. aureus*onceftriaxone biotransformation chromatogram(left). S = standard, E = extract, C = control

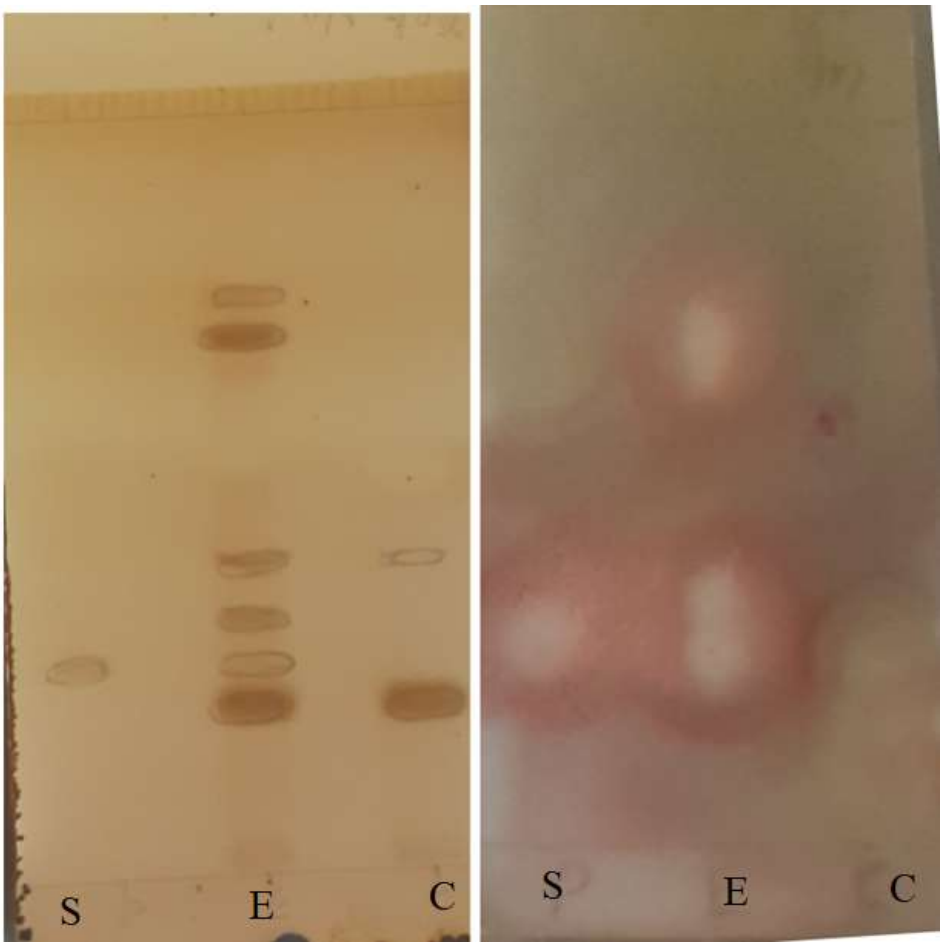


Figure 4.7: The bioautogram (right)of clinical *S. aureus*on ceftriaxone biotransformation chromatogram (left).

S = standard, E = extract, C = control

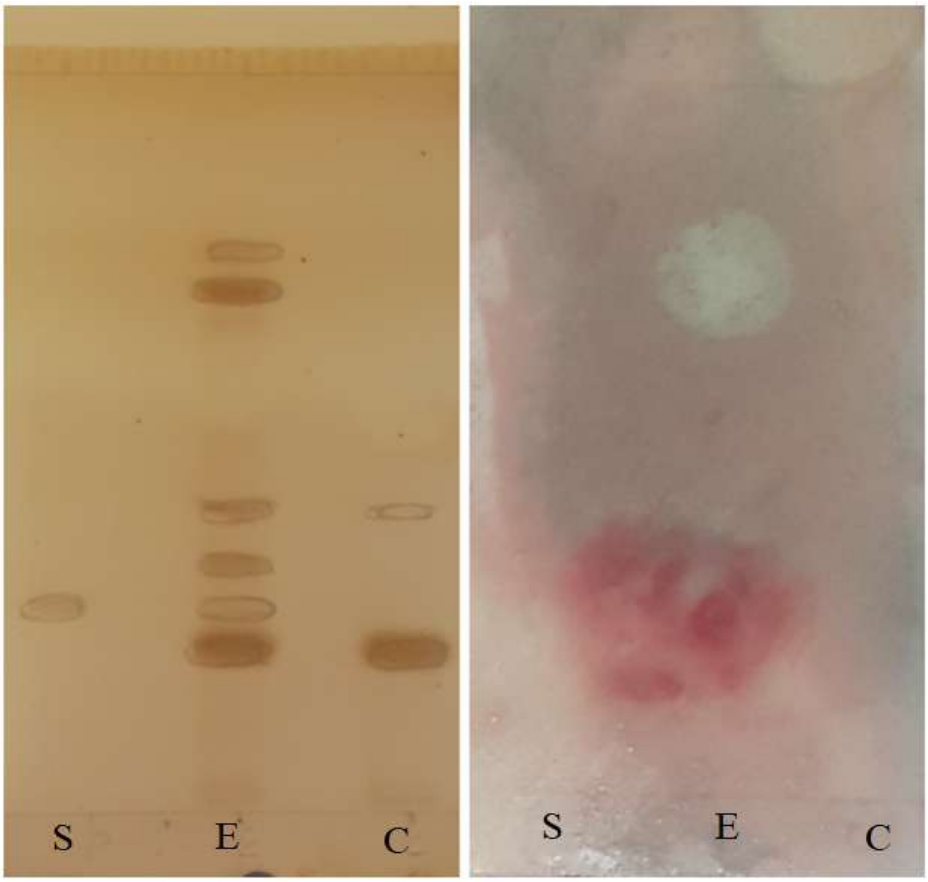


Figure 4.8: Bioautogram (right)of MRSA on ceftriaxone biotransformation chromatograms (left).

S = standard, E = extract, C = control

When tested on *E. coli*, the inhibition appeared to be as a result of the spot bands of parent antibiotic. The spots of new compounds did not appear to present antibacterial activity against both standard and clinical isolates of *E. coli* as shown in figure 4.9

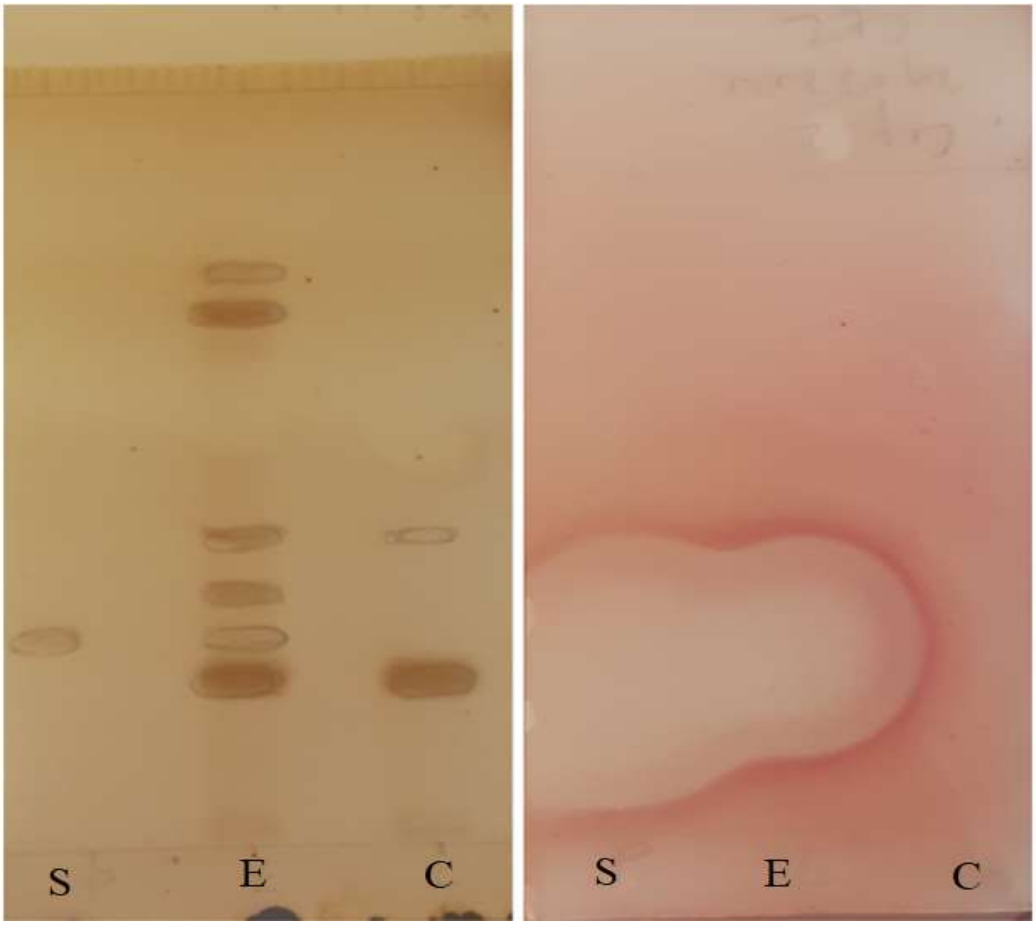


Figure 4.9: Bioautogram (right) of *E. coli* on ceftriaxone biotransformation chromatogram (left).

S = standard, E = extract, C = control

Also, when, ceftriaxone biotransformation chromatogram was tested on *Pseudomonas. aeruginosa* for antibacterial activity, only spots of the parent medicine were active only with faint inhibition zones. The spot for new compounds did not show any inhibition to both standard and clinical isolates (figure 4.10)

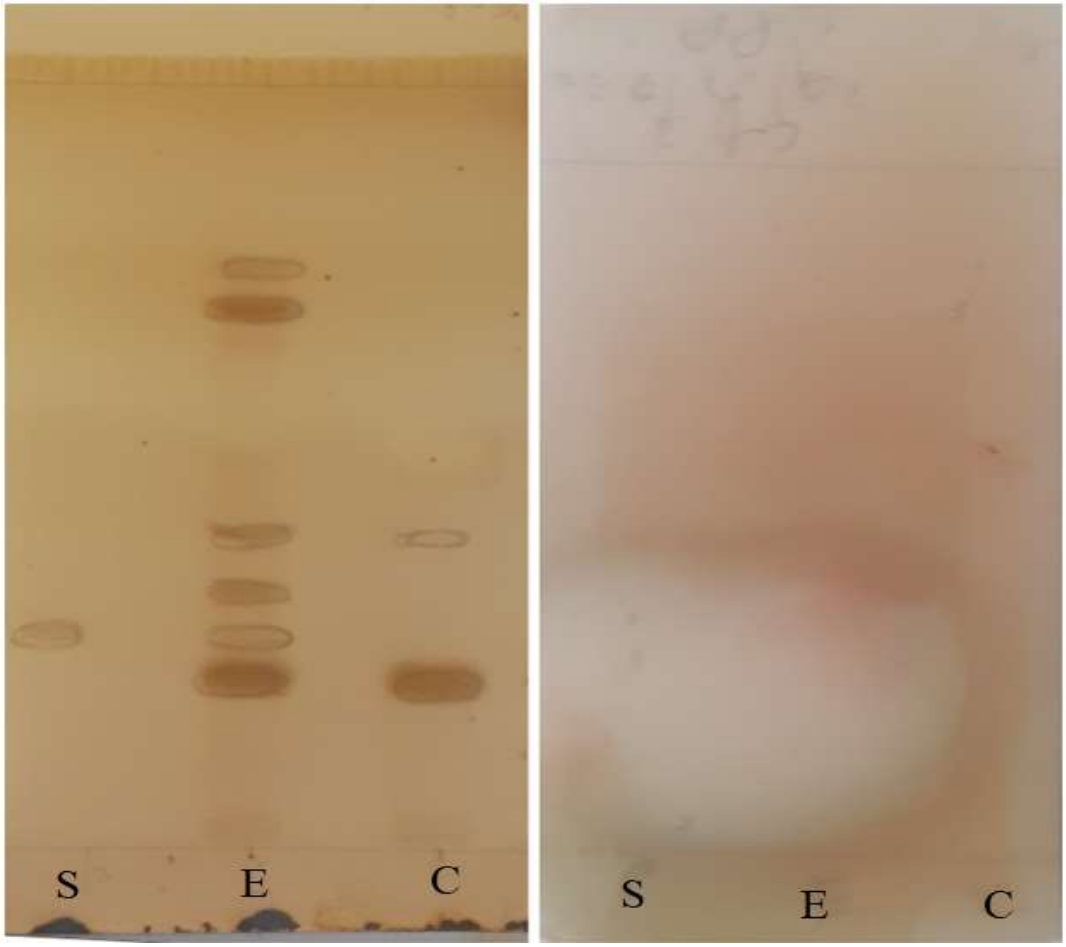


Figure 4.10: The bioautogram (right) of *P. aeruginosa* on ceftriaxone biotransformation chromatogram (left).

S = standard, E= extract, C= control

The biotransformation chromatograms of ciprofloxacin were also tested for antibacterial activities using agar overlay technique. Three bacteria were used as indicator organisms ie*S. aureus*, *E. coli* and *P. aeruginosa* both standard and clinical isolates. Results showed that most of the inhibition was due to the spot of parent drug rather than spot of a new compound except for bioautogram of standard *E. coli* whose inhibition also appeared to be centered at the spot of a newly formed compound (figure 4.11). Other bioautograms for *S. aureus* and *P.*

aeruginosa appeared centered on at the spot of parent drug (figure 4.12 and figure 4.13 respectively).

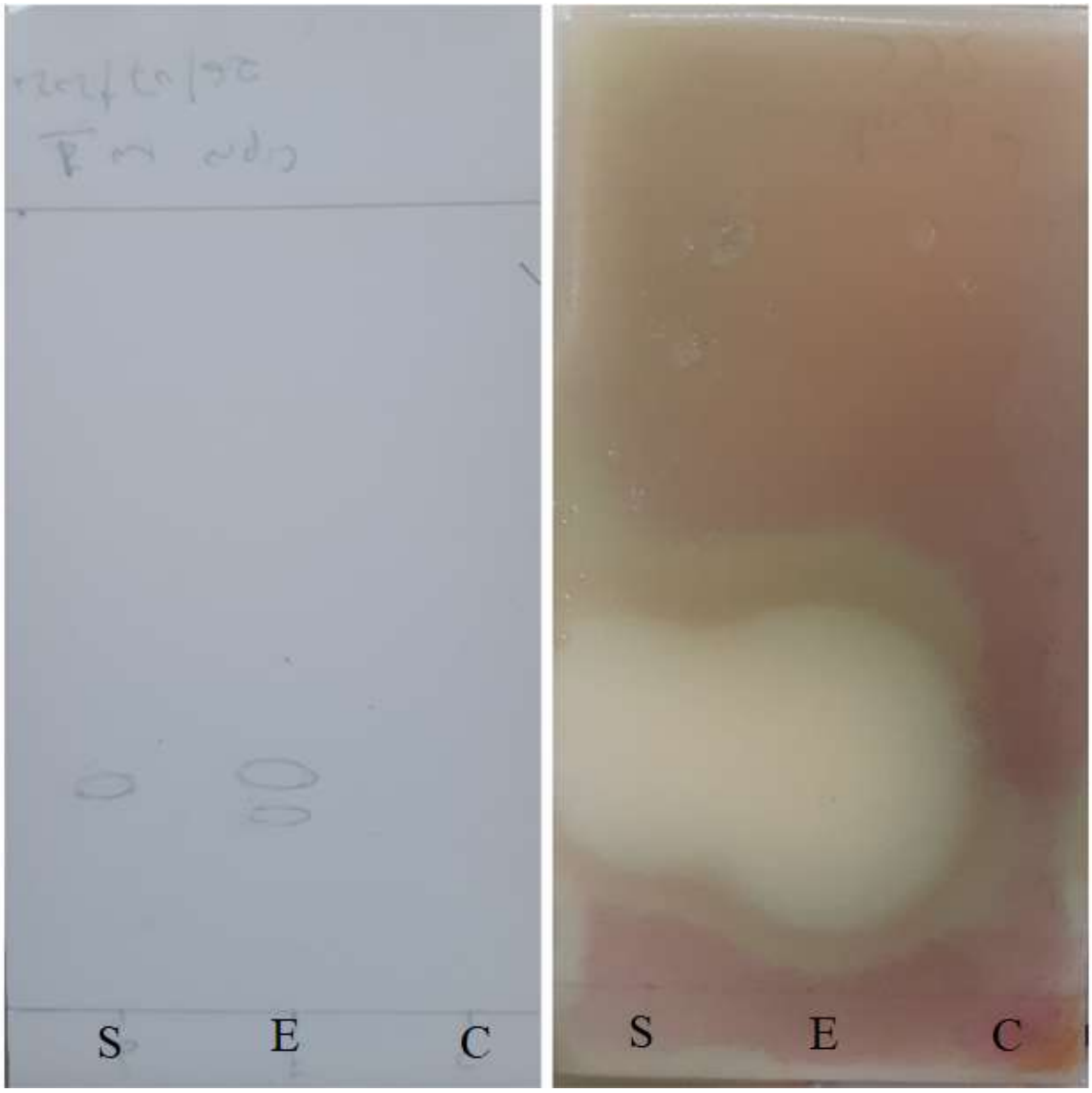


Figure 4.11: The bioautogram (right) of standard *E. coli* on ciprofloxacin biotransformation chromatogram (left).

S = standard, E = extract, C = control

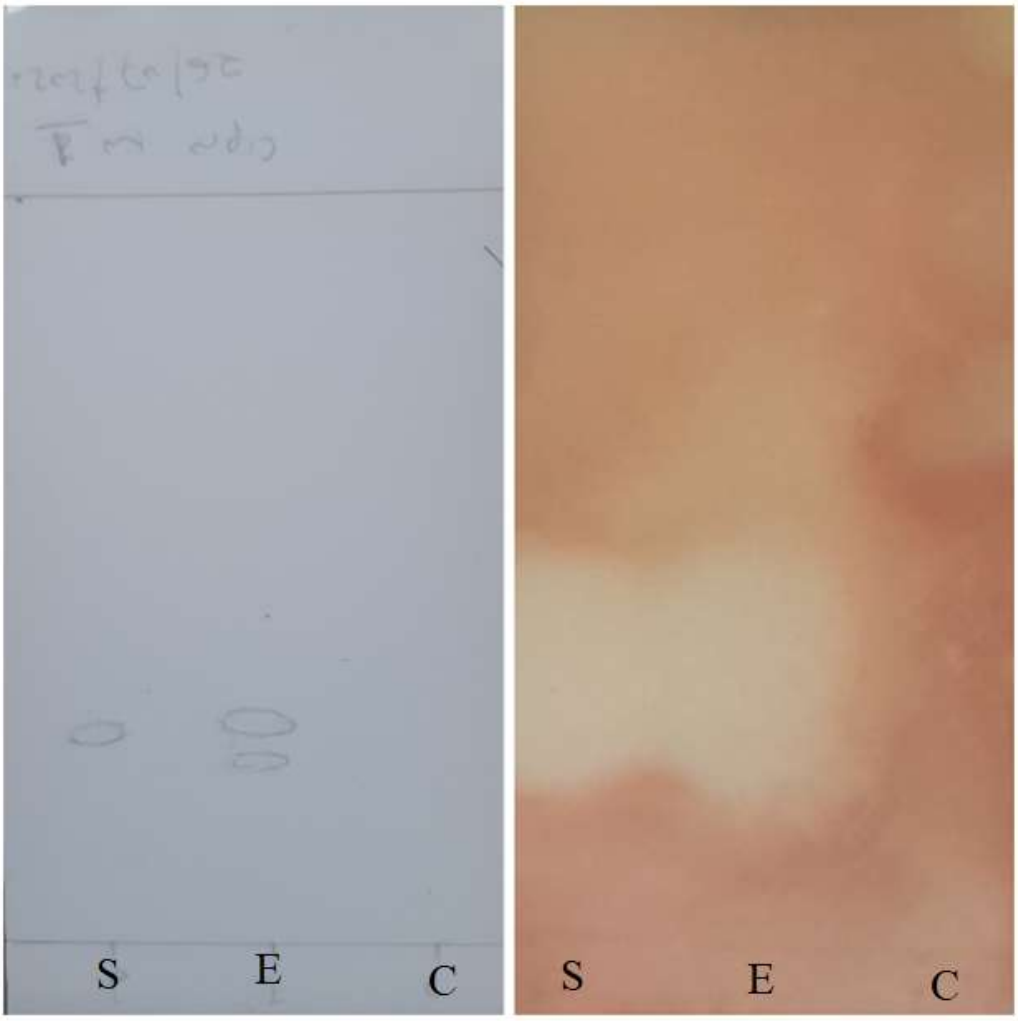


Figure 4.12: The bioautogram of standard *S. aureus* on ciprofloxacin biotransformation chromatogram.

S = standard, E = extract, C = control

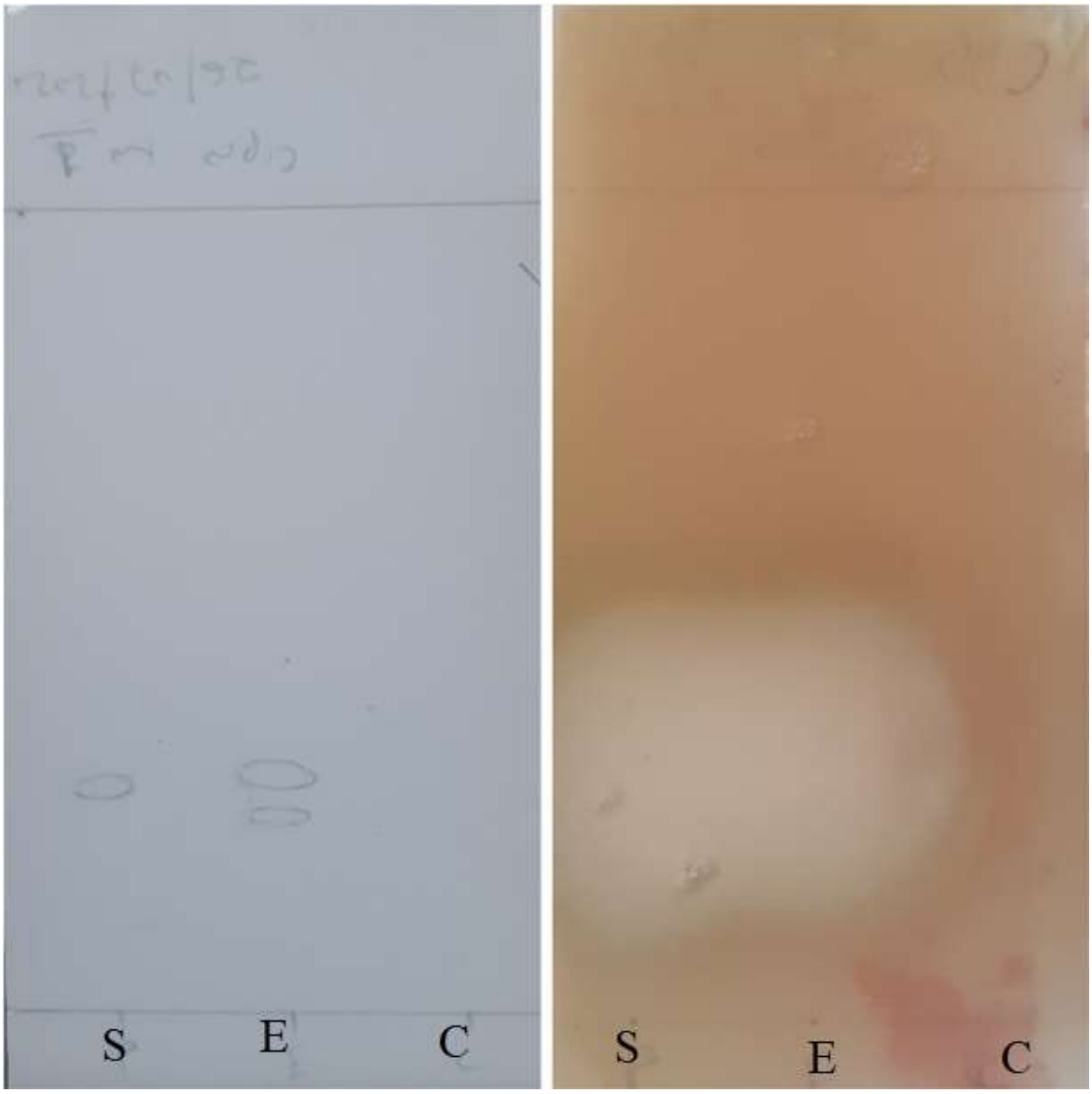


Figure 4.13: The bioautogram (right) of standard *P. aeruginosa* on ciprofloxacin biotransformation chromatogram (left).

S = standard, E = extract, C = control.

4.2 DISCUSSION

This study revealed the biotransformation potential of *Pleurotusostreatus* mushroom. This result is similar to a number of several other studies which have shown fungi including *P. ostreatus*to be one of most diverse groups of microorganism playing the key role of biotransformation[58,59]. For example, a study done byOlicon-Hernandez *et al* shows the role of P. ostreatus on ciprofloxacin (Figure 2.6 in section 2.2).

The number of mechanisms for biotransformation have been suggested including utilization of Cytochrome P450 system and soluble extracellular enzymes including lignin peroxidase, manganese peroxidase and laccase [60–62].

The study has indicated that *P. ostreatus* can act on single molecule and produce more than one compound. Biotransformation of ceftriaxone for instance has produced three new spots. This is however not different from several other biotransformation studies conducted elsewhere. For instance, a study conducted by Shah SAA *et al* demonstrated similar biotransformation results[39]. This could be possible due to presence of several active reaction sites on the compound and introduction of different substituents at different positions of the parent molecule.

Biotransformation of ciprofloxacin has indicated the formation of more a polar metabolite than the parent antibiotic. This is evidenced by lower Rf value of this metabolite (0. 26) as compared to that of parent antibiotic (0.39).

This finding is in agreement with most of suggested biotransformation mechanisms which result into formation of more polar metabolites than the parent compounds[20]. On the hand, biotransformation findings of ceftriaxone have shown formation of metabolites which are less polar than the parent compound. Several biotransformation pathways are possible, some important including hydroxylation, glycosylation, oxido-reductions between alcohols and ketones, hydrolysis, epoxidation, reduction of carbonyl groups, reduction of C-C double bonds and nitro reductions[63].

Bioautography has indicated that biotransformation of ceftriaxone and ciprofloxacin can produce new bioactive compound. From the bioautograms of *S. aureus* on ceftriaxone biotransformation chromatograms and bioautogram of standard *E. coli* on ciprofloxacin biotransformation chromatogram, the newly formed spots due to biotransformation appeared to be active.

This might have been possible due to presence of isosteres, that is, the removed group (substituent) and introduced group have similar biological properties. Biotransformation has been shown to be the source of lead compounds for drug discovery and development. There are other studies which have shown several pharmacologically active drugs which were obtained from biotransformation process for example penicillin G, cyclosporin, fusidic acid[38].

Nevertheless, the bioautographic analysis showed that in some cases, the spots for parent drugs were active while new spots were inactive. Despite some spots being regarded to be inactive against tested bacteria, the activities for some spots could have been missed due to lack of good resolution between spots of parent drug and new spots.

4.2.1 Study Limitations.

Despite the success obtained from this work, a number of challenges were encountered. One of the limitations which could have led to wrongful findings is unaware contamination. This could have arisen due interference from other laboratories activities which sharing of devices and working space is the common practice in the setting. This was however mitigated by ensuring the possible maximum level of cleanliness.

Nevertheless, due to poor resolution of inhibition zones between the spots, some antibacterial activities could have been misinterpreted. This would however been mitigated by fractionation and carry out the antibacterial activity test on individual fraction.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATION

5.1. CONCLUSION

The biotransformation of the known and commonly used antibiotics ceftriaxone and ciprofloxacin by *P.ostreatus* mushroom has yielded possibly new metabolites hence showed the biotransformation potential of *P. ostreatus*. The chromatographic analysis indicated three new compounds resulted from biotransformation of ceftriaxone and one from biotransformation of ciprofloxacin.

The bioautography of the new metabolites has indicated the presence of antibacterial activity comparable to that of parent compounds. This indicates that biotransformation by *P.ostreatus* mushroom can be a very useful alternative tool for searching new compounds which can be beneficial in drugs discovery and development.

From this study biotransformation has demonstrated its advantages over conventional synthetic approach as it is cheap, mild reaction conditions, environmentally friendly, and simple operation procedures. With that regard it can be used to carry out various syntheses which could not be possible with conventional synthesis.

5.2. RECOMMENDATION

The work which has been completed in this study is just a very minute fraction of the whole process of drug discovery and development. Much needs tobe done so as to get new medicines in the market which can be beneficial in treatment of resistant bug.

It is recommended that

- Further studies to be conducted to isolate the resulted compounds and endeavor to determine their structures.
- In addition, more work is needed to investigate the pharmacophore of the compounds and carry out all necessary structural modification which can help to improve the activity and reduce the deleterious effects which may arise.
- Furthermore, investigations should be performed forantibacterial activity of new potential compounds on many other bacterial species and in vivo studies to ascertain that they can be safely used. Additionally, the newly formed spots can also be investigated for other pharmacological activities such as antifungal activities, antitumor activities etc.
- Some spots could have been missed for the antibacterial activities due to lack of clear resolution between the spot for parent drug and spot of newly formed compound. So, it is called more work to be done to isolate the fractions and test for antibacterial activities separately. Moreover, the biotransformation potential of *Pleurotusostreatus* mushroom can be assessed on other classes of available medicines.

REFERENCES.

1. CDC. Achievements in Public Health, 1900-1999: Control of Infectious Diseases. 1600 Clifton Rd, MailStop E-90, Atlanta, GA 30333, U.S.A. 1999. p. 621–9.

2. Chokshi A, Sifri Z, Cennimo D, Horng H. Global contributors to antibiotic resistance. J Glob Infect Dis. 2019 Jan 1;11(1):36–42.

3. Ashley DJ, Brindle MJ. Penicillin resistance in staphylococci isolated in a casualty department. J Clin Pathol. 1960 Jul 1;13:336–8.

4. WHO. Prioritization of Pathogens to Guide Discovery, Research and Development of New Antibiotics for Drug-Resistant Bacterial Infections, Including Tuberculosis. Geneva, Switzerland; 2017.

5. Howard DH, Scott RD, Packard R, Jones D. The Global Impact of Drug Resistance. Clin Infect Dis. 2003;36(Supplement_1):S4–10.

6. Roberts RR, Scott RD, Hota B, Kampe LM, Abbasi F, Schabowski S, et al. Costs attributable to healthcare-acquired infection in hospitalized adults and a comparison of economic methods. Med Care. 2010 Nov;48(11):1026–35.

7. O'neill J. Tackling Drug-Resistant Infections Globally: Final Report and Recommendations; The Review on Antimicrobial Resistance . 2016.

8. O'neill J. Antimicrobial Resistance : Tackling a crisis for the health and wealth of nations. 2014;(December).

9. European study: 33,000 deaths a year from resistant infections | CIDRAP [Internet]. [cited 2019 Dec 7]. Available from: http://www.cidrap.umn.edu/news-perspective/2018/11/european-study-33000-deaths-year-resistant-infections

10. Shah NS, Wright A, Bai GH, Barrera L, Boulahbal F, Martín-Casabona N, et al. Worldwide emergence of extensively drug-resistant tuberculosis. Emerg Infect Dis. 2007;13(3):380–7.

11. Ndihokubwayo JB, Yahaya AA, Desta AT, Ki-Zerbo G, Odei EA, Keita B, et al. Antimicrobial resistance in the African Region: Issues, challenges and actions proposed [Internet]. [cited 2019 Dec 10]. Available from: http://www.who.int/bulletin/

12. Leopold SJ, van Leth F, Tarekegn H, Schultsz C. Antimicrobial drug resistance among clinically relevant bacterial isolates in sub-Saharan Africa: A systematic review. J Antimicrob Chemother. 2014;69(9):2337–53.

13. WHO. Draft global action plan on antimicrobial resistance. Geneva. 2015.

14. Taylor J, Hafner M, Yerushalmi E, Smith R, Bellasio J, Vardavas R, et al. Estimating the economic costs of antimicrobial resistance: Model and Results. 2050;

15. Hwang AY, Gums JG. The emergence and evolution of antimicrobial resistance: Impact on a global scale. Bioorganic Med Chem. 2016;24(24):6440–5.

16. Mauldin PD, Salgado CD, Hansen IS, Durup DT, Bosso JA. Attributable hospital cost and length of stay associated with health care-associated infections caused by antibiotic-resistant gram-negative bacteria. Antimicrob Agents Chemother. 2010 Jan;54(1):109–15.

17. Fair RJ, Tor Y. Antibiotics and bacterial resistance in the 21st century. Perspect Medicin Chem. 2014 Jun 24;(6):25–64.

18. de Kraker MEA, Davey PG, Grundmann H. Mortality and hospital stay associated with resistant Staphylococcus aureus and Escherichia coli bacteremia: Estimating the burden of antibiotic resistance in Europe. PLoS Med. 2011 Oct;8(10).

19. Mwambete KD, Nyaulingo B. Antibiotic Resistance Profiles of Bacterial Pathogens from Private Hospitals in Dar Es Salaam, Tanzania. 2014;6(2).

20. Borges KB, Borges W de S, Durán-Patrón R, Pupo MT, Bonato PS, Collado IG. Stereoselective biotransformations using fungi as biocatalysts. Vol. 20, Tetrahedron Asymmetry. Pergamon; 2009. p. 385–97.

21. Choudhary M ?Iqba., Shah S ?Adnan?Al., Sami A, Ajaz A, Shaheen F, Atta-ur-Rahman. Fungal Metabolites of (E)-Guggulsterone and Their Antibacterial and Radical-Scavenging Activities. Chem Biodivers [Internet]. 2005 Apr [cited 2019 Dec 9];2(4):516–24. Available from: http://doi.wiley.com/10.1002/cbdv.200590033

22. Illanes A, Cauerhff A, Wilson L, Castro GR. Recent trends in biocatalysis engineering. Bioresour Technol. 2012 Jul;115:48–57.

23. Baydoun E, Bibi M, Asif Iqbal M, Wahab A-T, Farran D, Colon S, et al. Microbial transformation of anti-cancer steroid exemestane and cytotoxicity of its metabolites against cancer cell lines. 2013.

24. Rozzell JD. Commercial scale biocatalysis: myths and realities. Bioorg Med Chem [Internet]. 1999 Oct [cited 2019 Dec 7];7(10):2253–61. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10579534

25. Zaks A, Dodds DR. Application of biocatalysis and biotransformations to the synthesis of pharmaceuticals. Drug Discov Today. 1997 Dec;2(12):513–31.

26. Venisetty R, Ciddi V. Application of Microbial Biotransformation for the New Drug Discovery Using Natural Drugs as Substrates. Curr Pharm Biotechnol. 2003 Jun 1;4(3):153–67.

27. Anne M Collins MJK. Biotransformations and Bioconversions in New Zealand: Past Endeavours and Future Potential. 1999 Dec 31;9(2):86–94.

28. Wiederschain GY. Stereoselective Biocatalysis. Vol. 66, Biochemistry (Moscow). 2001. p. 1187.

29. Grabarczyk M, Wińska K, MącZka W, Zarowska B, MacIejewska G, Dancewicz K, et al. Synthesis, biotransformation and biological activity of halolactones obtained from β-ionone. Tetrahedron. 2016;72(5):637–44.

30. Bernhardt R. Cytochromes P450 as versatile biocatalysts. J Biotechnol. 2006 Jun 25;124(1):128–45.

31. Hegazy MEF, Mohamed TA, ElShamy AI, Mohamed AEHH, Mahalel UA, Reda EH, et al. Microbial biotransformation as a tool for drug development based on natural products from mevalonic acid pathway: A review. Vol. 6, Journal of Advanced Research. Elsevier; 2015. p. 17–33.

32. Rozenbaum HF, Patitucci ML, Antunes OAC, Pereira N. Production of aromas and fragrances through microbial oxidation of monoterpenes. Brazilian J Chem Eng. 2006;23(3):273–9.

33. Yousuf M, Mammadova K, Baghirova S, Rahimova R. Mini Review Nov Appro Drug Des Dev Biotransformation : A One Pot Method of Biotransformation : A One Pot Method of Novel Pharmacological Importance. 2019;(October).

34. Liu JH, Chen YG, Yu BY, Chen YJ. A novel ketone derivative of artemisinin biotransformed by Streptomyces griseus ATCC 13273. Bioorganic Med Chem Lett. 2006 Apr 1;16(7):1909–12.

35. Zhan J, Guo H, Dai J, Zhang Y, Guo D. Microbial transformations of artemisinin by Cunninghamella echinulata and Aspergillus niger. Tetrahedron Lett. 2002;43(25):4519–21.

36. Lee IS, ElSohly HN, Croom EM, Hufford CD. Microbial metabolism studies of the antimalarial sesquiterpene artemisinin. J Nat Prod. 1989 Mar 1;52(2):337–41.

37. Zhang P, Lin LH, Huang HH, Xu HY, Zhong DF. Biotransformation of indomethacin by the fungus Cunninghamella blakesleeana. Acta Pharmacol Sin. 2006 Aug;27(8):1097–102.

38. Huisman GW, Gray D. Towards novel processes for the fine-chemical and pharmaceutical industries. Curr Opin Biotechnol. 2002 Aug;13(4):352–8.

39. Shah SAA, Sultan S, Zaimi Bin Mohd Noor M. Biotransformation of tissue-specific hormone tibolone with fungal culture Trichothecium roseum. J Mol Struct. 2013 Jun 24;1042:118–22.

40. Fernandes Â, Barros L, Martins A, Herbert P, Ferreira ICFR. Nutritional characterization of Pleurotus ostreatus (Jacq. ex Fr.) P. Kumm. produced using paper scraps as substrate.

41. Stojković D, Reis FS, Glamočlija J, Ćirić A, Barros L, Van Griensven LJLD, et al. Cultivated strains of Agaricus bisporus and A. brasiliensis: Chemical characterization and evaluation of antioxidant and antimicrobial properties for the final healthy product-natural preservatives in yoghurt. Food Funct. 2014;5(7):1602–12.

42. GBIF. Alces alces (Linnaeus, 1758) [Internet]. GBIF | Global Biodiversity Information Facility. GBIF Secretariat; 2016 [cited 2019 Dec 9]. p. Available from: https://www.gbif.org/species/2440940

43. Hofrichter M, Ullrich R, Pecyna MJ, Liers C, Lundell T. New and classic families of secreted fungal heme peroxidases. Vol. 87, Applied Microbiology and Biotechnology. Springer Verlag; 2010. p. 871–97.

44. Pointing SB. Feasibility of bioremediation by white-rot fungi. Vol. 57, Applied Microbiology and Biotechnology. 2001. p. 20–33.

45. Asgher M, Bhatti HN, Ashraf M, Legge RL. Recent developments in biodegradation of industrial pollutants by white rot fungi and their enzyme system. Biodegradation. 2008;19(6):771–83.

46. Buchicchio A, Bianco G, Sofo A, Masi S, Caniani D. Biodegradation of carbamazepine and clarithromycin by Trichoderma harzianum and Pleurotus ostreatus investigated by liquid chromatography - high-resolution tandem mass spectrometry (FTICR MS-IRMPD). Sci Total Environ. 2016 Jul 1;557–558:733–9.

47. Olicón-Hernández DR, González-López J, Aranda E. Overview on the biochemical potential of filamentous fungi to degrade pharmaceutical compounds. Front Microbiol. 2017;8(SEP):1–17.

48. Hamburger M, Hostettmann K. 7. Bioactivity in plants: the link between phytochemistry and medicine. Phytochemistry. 1991;30(12):3864–74.

49. Rahalison L, Hamburger M, Hostettmann K, Monod M, Frenk E. A bioautographic agar overlay method for the detection of antifungal compounds from higher plants. Phytochem Anal. 1991 Nov;2(5):199–203.

50. Shahverdi AR, Fakhimi A, Shahverdi HR, Minaian S. Synthesis and effect of silver nanoparticles on the antibacterial activity of different antibiotics against Staphylococcus aureus and Escherichia coli. Nanomedicine Nanotechnology, Biol Med. 2007 Jun;3(2):168–71.

51. Navarro N, Yúfera M. Population dynamics of rotifers (Brachionus plicatilis and Brachionus rotundiformis) in semicontinuous culture fed freeze-dried microalgae: Influence of dilution rate. Aquaculture. 1998 Jul 15;166(3–4):297–309.

52. Hamburger MO, Cordell GA. A Direct Bioautographic Tlc Assay for Compounds Possessing Antibacterial Activity. J Nat Prod [Internet]. 1987 Jan [cited 2019 Dec 10];50(1):19–22. Available from: https://pubs.acs.org/doi/abs/10.1021/np50049a003

53. Rios JL, Recio MC, Villar A. Screening methods for natural products with antimicrobial activity: A review of the literature. J Ethnopharmacol. 1988;23(2–3):127–49.

54. Edeoga HO, Okwu DE, Mbaebie BO. Phytochemical constituents of some Nigerian medicinal plants. African J Biotechnol. 2005;4(7):685–8.

55. Marston A. Thin-layer chromatography with biological detection in phytochemistry. Vol. 1218, Journal of Chromatography A. 2011. p. 2676–83.

56. Phytochemical Methods A Guide to Modern Techniques of Plant Analysis - A.J. Harborne - Google Books.

57. Wińska K, Grabarczyk M, Mączka W, Żarowska B, Maciejewska G, Dancewicz K, et al. Biotransformation of Lactones with Methylcyclohexane Ring and Their Biological Activity. Appl Sci [Internet]. 2016 Dec 24 [cited 2019 Dec 11];7(1):12. Available from: http://www.mdpi.com/2076-3417/7/1/12

58. Mueller GM, Schmit JP, Leacock PR, Buyck B, Cifuentes J, Desjardin DE, et al. Global diversity and distribution of macrofungi. Biodivers Conserv [Internet]. 2007 Jan 27 [cited 2020 Aug 5];16(1):37–48. Available from: https://link.springer.com/article/10.1007/s10531-006-9108-8

59. Peng RH, Xiong AS, Xue Y, Fu XY, Gao F, Zhao W, et al. Microbial biodegradation of polyaromatic hydrocarbons [Internet]. Vol. 32, FEMS Microbiology Reviews. FEMS Microbiol Rev; 2008 [cited 2020 Aug 5]. p. 927–55. Available from: https://pubmed.ncbi.nlm.nih.gov/18662317/

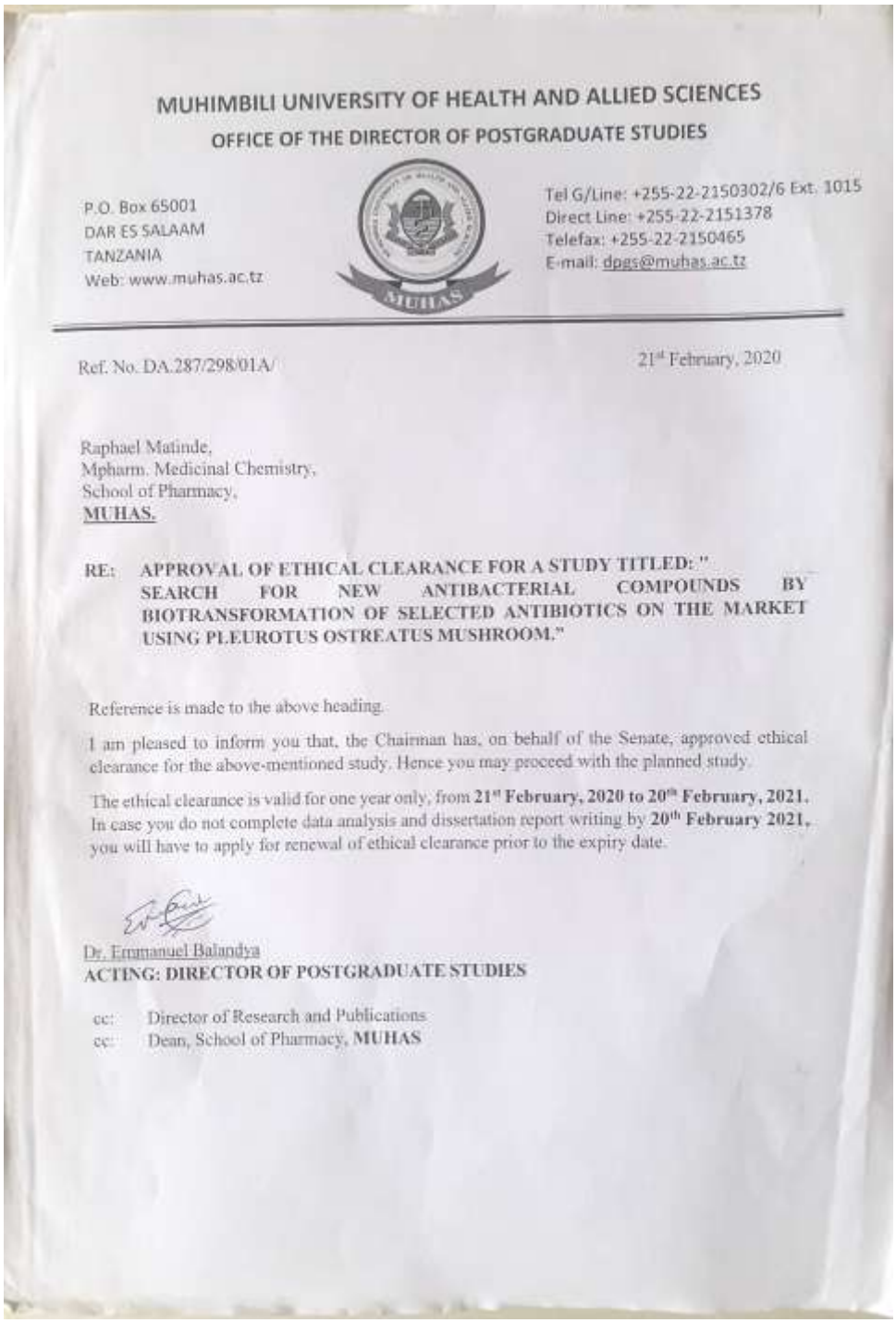
60. Yadav JS, Doddapaneni H, Subramanian V. P450ome of the white rot fungus Phanerochaete chrysosporium: Structure, evolution and regulation of expression of genomic P450 clusters. In: Biochemical Society Transactions [Internet]. Biochem Soc Trans; 2006 [cited 2020 Aug 5]. p. 1165–9. Available from: https://pubmed.ncbi.nlm.nih.gov/17073777/

61. Gianfreda L, Xu F, Bollag JM. Laccases: A useful group of oxidoreductive enzymes. Bioremediat J [Internet]. 1999 [cited 2020 Aug 5];3(1):1–26. Available from: https://www.tandfonline.com/doi/abs/10.1080/10889869991219163

62. Steffen KT, Hatakka A, Hofrichter M. Degradation of benzo[a]pyrene by the litter-decomposing basidiomycete Stropharia coronilla: Role of manganese peroxidase. Appl Environ Microbiol [Internet]. 2003 Jul 1 [cited 2020 Aug 5];69(7):3957–64. Available from: http://aem.asm.org/

63. Giri A, Dhingra V, Giri CC, Singh A, Ward OP, Narasu ML. Biotransformations using plant cells, organ cultures and enzyme systems: Current trends and future prospects. Biotechnol Adv. 2001 Jun 1;19(3):175–99.

Approval of Ethical Clearance



MUHIMBILI UNIVERSITY OF HEALTH AND ALLIED SCIENCES

OFFICE OF THE DIRECTOR OF POSTGRADUATE STUDIES

P.O. Box 65001
DAR ES SALAAM
TANZANIA
Web: www.muhas.ac.tz

Tel G/Line: +255-22-2150302/6 Ext. 1015
Direct Line: +255-22-2151378
Telefax: +255-22-2150465
E-mail: dpgs@muhas.ac.tz

Ref. No. DA.287/298/01A/

21st February, 2020

Raphael Matinde,
Mpharm. Medicinal Chemistry,
School of Pharmacy,
MUHAS.

RE: APPROVAL OF ETHICAL CLEARANCE FOR A STUDY TITLED: " SEARCH FOR NEW ANTIBACTERIAL COMPOUNDS BY BIOTRANSFORMATION OF SELECTED ANTIBIOTICS ON THE MARKET USING PLEUROTUS OSTREATUS MUSHROOM."

Reference is made to the above heading.

I am pleased to inform you that, the Chairman has, on behalf of the Senate, approved ethical clearance for the above-mentioned study. Hence you may proceed with the planned study.

The ethical clearance is valid for one year only, from **21st February, 2020 to 20th February, 2021.** In case you do not complete data analysis and dissertation report writing by **20th February 2021,** you will have to apply for renewal of ethical clearance prior to the expiry date.

Dr. Emmanuel Balandya
ACTING: DIRECTOR OF POSTGRADUATE STUDIES

cc: Director of Research and Publications
cc: Dean, School of Pharmacy, **MUHAS**