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Antimicrobial and cytotoxicity activities of conyza bonariensis, tribulus terrestris and rubia cordifolia growing in Arusha, Tanzania

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ANTIMICROBIAL AND CYTOTOXICITY ACTIVITIES OF *Conyza bonariensis*, *Tribulus terrestris* AND *Rubia cordifolia* GROWING IN ARUSHA, TANZANIA

Ambrose Antony Kiang'u Ghwanga

A Dissertation Submitted in Partial Fulfilment of the Requirements for the Degree of Master's in Life Sciences of the Nelson Mandela African Institution of Science and Technology

Arusha, Tanzania

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ABSTRACT

The aim of this study was to evaluate antimicrobial, synergistic and cytotoxicity activities of *Conyza bonariensis*, *Tribulus terrestris* and *Rubia cordifolia* growing in Tanzania. Two fungal strains, four Gram-negative bacteria and one Gram-positive bacterium all being human pathogens were tested using 96 well microdilution method. The minimum inhibitory concentrations (MICs) was used to determine antimicrobial and synergistic activity of the extracts whereas LC_{50} values on brine shrimp larvae *(Artemia salina)* were used to determine cytotoxicity of the plants extracts. About 14% of all uncombined extracts (224) demonstrated moderate antimicrobial activity with MIC value range of 0.7825 mg/mL - 1.5625 mg/mL. Synergistic effect was evaluated by treating the microbes by mixture of extracts. A binary combination of extracts had synergistic effect in all combinations and MIC for each microbe was lowered. About 44% of mixed extracts showed moderate antimicrobial activity with MIC value range of 0.7825 mg/mL - 1.5625 mg/mL. Strong antimicrobial activity of 0.3906 mg/mL was recorded for CBLC-CBLE, CBLC-CBRC and CBLC-TLC against *E. coli* and *S. typhi*. The comparison between MIC of plant extracts was done using analysis of variance (ANOVA) and differences among the means were determined for significance at P = 0.05 using statistical package for social science. The order of antimicrobial effectiveness of the plants was *C. bonariensis* > *T.terrestris* > *R. cordifolia* with average MIC 7.49, 10.2 and 13.6, respectively for all microbes. Root extracts and extracts extracted by ethyl acetate were the most effective plant part and solvent, respectively. *Candida albicans* and *Salmonella typhimurium* were the least sensitive fungus and bacterium to most of unmixed extracts. All extracts were non-toxic to brine shrimps except TTC, RCA *and* CBM with mildly toxicity of LC_{50} 52.5069, 75.8198 and 79.0076, respectively. Pharmacologists and herbalists should consider synergism of plant extracts for prescription of herbal medicines.

Key words: Antimicrobial, extract, MIC, synergistic effect, toxicity.

DECLARATION

I, Ambrose Antony Kiang'u Ghwanga do hereby declare to the Senate of the Nelson Mandela African Institution of Science and Technology that this dissertation is my own original work and that it has neither been submitted nor being concurrently submitted for degree award in any other institution.

25.03.2019

Ambrose A. K. Ghwanga

Date

Name and Signature of candidate

The above declaration is confirmed

25.03.2019

Dr. Musa Chacha

Date

Name and Signature of Supervisor

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CERTIFICATION

This is to certify that the accompanying dissertation by Ambrose Antony Kiang'u Ghwanga has been accepted in partial fulfilment of the requirement for the Degree of Master's in Life Science of the Nelson Mandela African Institution of Science and Technology Arusha, Tanzania.

Dr. Musa Chacha
Supervisor

25.03.2019
Date

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DEDICATION

This dissertation is dedicated first to the Almighty God and then to my family (my wife Jesca and our children Baraka, Tumaini and Faraja) for their love during my studies. Also to my parents for their mannered upbringing which I am cherishing today.

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LIST OF ABBREVIATIONS

MIC	Minimum inhibitory concentration
ANOVA	Analysis of variance
LC_{50}	Lethal concentration required to kill 50% of the population
HIV/AIDS	Human Immune Deficiency Virus/ Acquired Immune Deficiency Syndrome
MRSA	Methicillin-resistant *Staphylococcus aureus*
WHO	The World Health Organization
INT	Para-iodonitrotetrazolium chloride dye
DMSO	Dimethyl sulfoxide
UK	United Kingdom
LTD	Limited
ATCC	American Type Culture Collection
MUHAS	Muhimbili University of Health and Allied Sciences
TPRI	Tropical Pesticides Research Institute
g	Gram
mg	Milligram
µg	Microgram
^{o}C	Celsius
mL	Milliliter
µL	Microliter
%	Percent
CI	Confidence Interval
R^2	Retention Factor

CHAPTER ONE

INTRODUCTION

1.1 Background information

Infectious diseases remain to be one of the serious health and economic challenge in the world and particularly in developing countries (Funk *et al.*, 2005). They are the main cause of mortality and morbidity in the world causing about 43% of total deaths in Africa (Verma andf Singh, 2008; Issa *et al.*, 2018). Interaction between humans and animals and between human populations spreads microbial infections. The increase of drug resistant strains of pathogens and emergence of the immune-suppressed human population have highly contributed to the situation (Funk *et al.*, 2005; Verma and Singh, 2008; Bukhari *et al.*, 2013; Muhammad *et al.*, 2016). Also improper use of microbial drugs, antibiotics and antifungals, have reduced drug effectiveness against pathogenic bacteria and fungi leading to infection recurrence, failure to heal or extended healing (Leekha *et al*., 2011; Fair and Tor, 2014; Roca *et al*., 2015). The management of microbial infections, both synthetic and traditional medicines are used whereby about 80% use traditional medicine worldwide (Davis *et al.*, 2009; Dastagir *et al.*, 2012; Hashim *et al.*, 2014; Issa *et al.*, 2018). Traditional medicine has become a reliable source of antimicrobial drugs (Mahmood *et al.*, 2011, Dastagir *et al.*, 2012; Thabit *et al.*, 2014; Muhammad *et al.*, 2016; Kibonde *et al.*, 2018), due to its effectiveness and long existence.

In vitro testing of medicinal plants helps to understand their antimicrobial potencies against selected pathogenic microbes. Cytotoxicity levels usually on *Artemia salina* primarily determine the safety of plants on human cells (Meyer *et al., 1982*). Fungal infections such as Candidiasis caused by mainly *Candida albicans* in Africa and meningoencephalitis caused by *Cryptococcus neoformans* are common to immunosuppressed populations such as those with HIV/AIDS and with diabetes mellitus (Massimo and Naidu, 2015; Rajasingham *et al*., 2015). Gram-negative pathogenic bacteria such as *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium* and *Salmonella typhi* also cause troublesome bacterial infections (Russo and Johnson, 2000; Haghjoo and Galán, 2004; Meurens *et al*., 2009; Tacconelli *et al*., 2014). *Staphylococcus aureus* is a clinically important Gram-positive bacteria due to its invasive trend to people with low immunity and its rising resistance to methicillin antibiotics (Tacconelli *et al*., 2014).

Tanzania has high diversity of medicinal plants which when studied can pave the path for modern drug production in the country. Tibandebage *et al*. (2016) inform that the production of pharmaceuticals in Tanzania has witnessed a considerable down fall since 2006. They further report that in 2004-2005, Tanzania had seven (7) pharmaceutical firms that were manufacturing human medicines in Tanzania. At this time, there were no multinational producers but only one joint venture with an external partner. The country experienced shutdown of pharmaceutical companies in 2008-2009 when both the number of local producers and medicine product variety dropped considerably because all local producers including the only anti-retroviral producer were closed by 2013 except one producer of basic antibiotics that survived (Tibandebage *et al.*, 2016). Searching of possible drug precursors and drug production has to be revived to save and acquire forex from importation and exportation, respectively. The emergence of multi-drug resistant pathogens, unaffordability and unavailability of proper medicine to poor societies also call for searching for new safe and effective antimicrobial drugs (Bukhari *et al.*, 2013).

Medicinal plants contain secondary metabolites such as monoterpenes, diterpenes, triterpenes, tetraterpenes, sesquiterpenes, saponnins, flavonoids, steroids and coumarins which have antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory and antimuscidal activities (Paiva *et al*., 2010; Shinkafi, 2014). Traditional medicine emanating from medicinal plants is more preferred than synthetic medicines (Innocent *et al*., 2014; Tibandebage *et al.*, 2016). About 60% Tanzanians depend on medicinal plants to treat microbial infections (Joseph *et al*., 2007). For example, Chagga, Sukuma and Kurya use *C. bonariensis* and *T. terrestris* among others for treatments of microbial ailments such as skin infections, toothache and in wounds healing. Rwandese refugees in Kagera and Indians in Arusha use *R. cordifolia* to cure syphilis, gonorrhea, toothache and skin infections (Ramathal and Ngassapa, 2001).

Although Tanzanian *C. bonariensis, T. terrestris* and *R. cordifolia* are among traditionally used plants to cure different infections in some communities, their medicinal properties have not yet been studied. Therefore, this study intends to evaluate antimicrobial potencies, synergistic and cytotoxicity activities of *C. bonariensis, T. terrestris* and *R. cordifolia* growing in Tanzania.

1.2 Research problem and justification

Fungal and bacterial infections are a common culprit in human health especially in developing countries and Africa in particular (Karat *et al*., 2016). Human morbidity and mortality cases due to microbial infections are common problems in poor communities due to poor health services. The pathogenic fungi and bacteria have acquired adaptive mechanisms to antimicrobial drugs elevating prevalence of microbial infections (Arif *et al.*, 2009; Srinivasan *et al.*, 2014). Consequently, microbial drug resistance has challenged the availability of effective antimicrobial therapies in developing countries. However, most of developing countries have plenty of medicinal plants used traditionally to cure infectious diseases.

Most people in Tanzania use medicinal plants to treat microbial infections and manage health disorders (Stangeland *et al.*, 2008; Stanifer *et al.*, 2015). Nevertheless, very few medicinal plants have been studied for drug production. *In vitro* revelation of medicinal efficacies of plants has been a crucial step in identification of lead drugs. The study evaluates antifungal, antibacterial, cytotoxicity as well as the antimicrobial synergistic effect of extracts. Validation of ethno-medical information about *C. bonariensis*, *T. terrestris* and *R. cordifolia* are the benefits of the study. Also the baseline for further studies on drug discovery and sensitization on conservation and commercialization of medicinal plants signify the study.

1.3 Significance of the study

This study has validated the ethno medical information pertaining to the use of *C. bonariensis*, *T. terrestris* and *R. cordifolia* to cure microbial infirmities in Tanzania. This study adds value on current efforts of searching for effective drugs to cure both emerging infections and drug resistant infections by assessing the medicinal values of Tanzanian *C. bonariensis*, *T. terrestris* and *R. cordifolia* that are locally used to treat microbial ailments. The need for conservation and commercialization of the studied plants is advocated by the findings from this study.

1.4 General objective

The main objective of this study was to evaluate antimicrobial and cytotoxicity activity of Tanzanian *C. bonariensis*, *T. terrestris* and *R. cordifolia.*

1.5 Specific objectives

(i) To evaluate antifungal and antibacterial activity of Tanzanian *C. bonariensis*, *T. terrestris* and *R. cordifolia* extracts against the selected bacterial and fungal strains.

(ii) To evaluate the synergistic effect of selected extracts against selected bacterial and fungal strains.

(iii) To determine cytotoxicity of Tanzanian *C. bonariensis*, *T. terrestris* and *R. cordifolia* extracts against brine shrimps larvae.

1.6 Research questions

(i) What are the antimicrobial potencies of Tanzanian *C. bonariensis*, *T. terrestris* and *R. cordifolia* on the selected bacteria and fungi?

(ii) Do bioactive compounds from plant extracts have synergistic or antagonistic effect against bacterial and fungal strains?

(iii) What are the toxicity levels of *C. bonariensis*, *T. terrestris* and *R. cordifolia* extracts against brine shrimps larvae?

CHAPTER TWO

LITERATURE REVIEW

2.1 History of antimicrobial drug development

The prime aim of the first generation of antimicrobial drugs was to save lives lost from lethal antimicrobial infections (Powers, 2004). The development of antifungals and antibiotics occurs in phases and new synthesis meant to address the weakness of its predecessor. Polyene and azole which are sensitive to toxins in human body and not suitable to patients in multidrug therapy, are replaced by echinocandins (Pfaller, 2012; Srinivasan *et al*., 2014; Balkovec *et al*., 2014). Also a third generation cephalosporin antibiotic is more effective against Gram-negative bacteria than the first generation cephalosporin (Powers, 2004). It is easier to develop antibiotics than antifungals because both fungal and human cells are eukaryotic and thus share more similarities than bacterial cells which are prokaryotic (Srinivasan *et al*., 2014)

2.2 Antimicrobial resistance against infectious diseases

Drug resistance is defined as the situation when the bacterial or fungal growth is not inhibited by the normal dosage of the drug and that the clinical drug efficacy against the pathogen has been reliably reduced (Pfaller, 2012). Antimicrobial resistance has been a big worldwide concern because it reduces the ability of drugs to cure fatal diseases (Powers, 2004)**.** Drug resistance against infectious diseases began even before the discovery of penicillin (Abraham and Chain, 1988). Almost all pathogenic bacteria have evolved their β-lactamases to become drug resistant to antibiotics used against them in treatment (Laxminarayan *et al*., 2013). In the past decade, microbial drug resistance was so critical to the extent that the World Health Organization described it as growing world health delinquent issue (Roca *et al*., 2015). Nanomaterial have been reported to produce a dominant possibility in production of effective drugs against multiple drug resistance pathogens (Aruguete *et al*., 2013). However, the drugs from nanomaterial may be expensive to low income earners and thus efforts invested on using phytochemicals which have shown strong antimicrobial activities against multiple resistance pathogens in production affordable drugs against the drug resistant pathogens (Moreno *et al*., 2015).

2.3 Mechanisms of drug resistance

Several mechanisms of pathogens to acquire drug resistance have been reported (Pfaller, 2012; Srinivasan *et al*., 2014). These include the induction of efflux pumps, which lead to decreased drug concentration at the enzyme target within the pathogen cell and acquisition of point mutations in the gene responsible for encoding the target enzyme when the pathogen has been exposed to the drug. Other mechanisms are the prevention of the drug at the cell membrane/cell wall level, inhibition of host cell to secret enzymes that activate the drug, and secretion of enzymes that degrade the drug to the extracellular medium. Microorganisms can also acquire drug resistance via acquisition of mobile genetic materials carrying drug resistant genes and occurrence of mutations even when the patient is not exposed to antimicrobial agents (Roca *et al*., 2015). The exposure of microorganisms to antimicrobial agents gives the necessary selective pressure for the rise and spread of the resistant pathogens (Roca *et al*., 2015; Levin-Reisman *et al*., 2017). Therefore, when antibacterial agents are misused or abused in humans, livestock and even in the environment increase the rate of microbial resistance (Laxminarayan *et al*., 2013; Roca *et al*., 2015). While high rates of using antibiotics in developed countries has been reported to enable the persistence of resistant strains, in developing countries the use of antibiotics is reported to increase with the increase in incomes, high rates of hospitalization and high prevalence of hospital infections (Laxminarayan *et al*., 2013). Furthermore, non-growing or slow growing bacteria acquire drug resistance when exposed to antimicrobial agents which kill the actively growing bacteria (Levin-Reisman *et al*., 2017). It is further reported that the production of antimicrobial drugs has decreased worldwide because there are few precursor drugs to develop (Powers, 2004).

2.4 Pathogenic microbes responsible for infectious diseases

The World Health Organization (WHO) defines infectious diseases as the diseases caused by pathogenic microorganisms, such as bacteria, viruses, parasites or fungi; that can directly or indirectly be transmitted from one person to another. Although infectious diseases are only leading major cause of deaths in low and middle income countries, they are still a major concern all over the world (Dye, 2015). According to the world health organization report, only about 20 species among 1400 species of pathogenic microbes cause two-thirds of deaths worldwide (Woolhouse and Gowtage-Sequeria, 2005).

Apart from vaccination with potentials to eradicate some infectious and presence of antimicrobial drugs, the fight against infectious diseases is not yet done mainly due to microbial resistance to drugs and scarcity of new antimicrobial drugs (Dye, 2015).

Candida species including *C. albicans* cause invasive candidiasis which is reported as the most common fungal disease in the world. Candidiasis affects higher than 250 000 people killing around 40% of patients including those receiving antifungal treatment since the pathogen has acquired resistance to present antifungals (Kullberg and Arendrup, 2015). In about 15 Candida species, *C. albicans* is among the five most virulent causing more than 90% of candidiasis worldwide (Pappas *et al*., 2015). Application of traditional medicine in curing candidiasis is justified because infections are easily identified and positive changes are clearly seen during treatment. Most traditional healers are reported to use roots, leaves, roots and barks, and stem barks of medicinal plants more than fruits and whole plants (Mayoi, 2014; Masevhe *et al*., 2015).

Cryptococcus neoformans is a human fungus that causes meningoencephalitis mainly to immunosuppressed communities like people with AIDS and approximated to cause 625 000 deaths yearly worldwide (Park *et al*., 2009). It is assumed that the first encounter to Cryptococci occurs in the lungs whereby macrophages and adaptive immunity response collectively clears the pathogens in immune-competent individuals (Shibuya *et al*., 2005). Cryptococcosis is ranked third in causing deaths of HIV/AIDS victims ahead of tuberculosis (Park *et al*., 2009).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a Gram positive bacterium. Gram positive are distinguished from Gram negative bacteria basing on the structure of their cell walls because Gram positive lack a thick outer layer of peptidoglycan on their cell walls and when treated with dyes, retain the colour of the dye (Gregersen, 1978). Multi drug resistant Gram-negative bacteria increasingly synthesize extended spectrum b-lactamase and carbapenemases to resist even the third-generation cephalosporins antibiotics (Tacconelli *et al*., 2014). *Staphylococcus aureus* bacterium causes fatal necrotizing fasciitis, a severe skin, subcutaneous tissue and superficial fascia occurring mostly in the limbs and in the abdominal walls (Headley, 2003; Chhetry *et al*., 2016). The pathogen is the cause for community-acquired and nosocomial infections apart from netrotizing fasciitis such as infective endocarditis, pneumonia and toxic shock syndrome (Wertheim *et al*., 2005; Shaikh *et al*., 2018).

The intrinsic virulence factors of the MRSA, its nasal carriage and the capacity to adapt easily to different environmental conditions enable MRSA to be transmitted easily and can cause repeated infections (Shaikh *et al*., 2018).

Escherichia coli is a common commensal flora in humans, other mammals and birds despite that is an important cause of bacterial infections. Only about 20% of *E. coli* strains are seriously virulent causing intestinal infections (Russo and Johnson, 2000). The pathogen is transmitted via person- to- person transmission directly or through fecal-oral route or through intake of contaminated food and/ or water (Scott and Bloomfield, 1990).

Pseudomonas aeruginosa is also an antibiotic resistant Gram-negative bacterium reported to occur in many countries (Tacconelli *et al*., 2014). Pseudomonas infections are reported among patients with organ transplant, acute leukemia, intravenous-drug addiction, burn wounds and cystic fibrosis (Bodey *et al*., 1983). The pathogen is nosocomial found in water taps, sink and to other hospital water sources being transmitted from patients to water systems and vice versa (Loveday *et al*., 2014; Garvey *et al*., 2016).

Salmonella typhimurium causes fatal food-born gastrointestinal bacteremia to immunocompromised patients (Lathrop *et al*., 2015). Transmission of *S. typhimurium* occurs when humans consume chicken and pork products contaminated with the pathogen (Prendergast *et al*., 2009; Pande *et al*., 2016). In pigs, the pathogen invades both small and large intestine leading to diarrhea and seldom sepsis but mostly infections produce no symptoms (Meurens *et al*., 2009). The antimicrobial resistance of *S. typhimurium* is reported to have been contributed by the overuse of antibiotics in growth boosters in animals (Nagid *et al*., 2015)

Salmonella typhi causes lethal typhoid fever, a public health trouble for a long time especially in low and middle income countries (Deen *et al*., 2012). *Salmonella typhi* pathogen is host-specific to human beings (Haghjoo and Galán, 2004; Galán, 2016). The pathogen produces exotoxin called cytolethal distending toxin only when it parasitizes human cells accounting for its specific pathogenesis to human and its peculiar symptoms (Haghjoo and Galán, 2004). Multi-drug resistance of *S. typhi* to antimicrobials began in 1970s and 1980s where ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole could no longer cure typhoid fever and replaced by Fluoroquinolones antimicrobials (Deen *et al*., 2012; Thanh *et al.*, 2016). Crump and Mintz (2010) approximate new cases of typhoid fever to be 20-30 million globally per year.

2.5 Medicinal plants commonly used for treatment of infectious diseases in Tanzania

According to WHO (2004), traditional medicine involves the use of experience and observations in management of health conditions transmitted from one generation to another either in writing or orally. Like in many developing countries, traditional medicine is preferred by most Tanzanians. A survey conducted in the northern part of the country learnt that, about 56% of people used traditional medicines of whom 76% were females and 68% of respondents knew someone using traditional medicine (Stanifer *et al.*, 2015). Therefore, traditional medicine plays a big role in the country and should be well documented and communicated. It covers the gap of insufficient conventional medicines in the country of which its production has experienced a steady decline (Stangeland *et al.*, 2008). Revelation of medicinal properties of the plants to the public is necessary to benefit more people and insight the need for drug formulation. The common medicinal plants used in Tanzania include *C. bonariensis*, *T. terrestris* and *R. cordifolia.*

2.6 Conyza bonariensis, Tribulus terrestris and Rubia cordifolia

Conyza bonariensis, *T. terrestris* and *R. cordifolia* belong to families Asteraceae, Zygophyllaceae and Rubiaceae, respectively. Asteraceae is the largest plant family with 1600-1700 genera carrying 24 000-30 000 species distributed in all continents except Antarctica (Funk *et al.*, 2005). The genus Conyza possesses secondary metabolites such as flavonoids, terpenoids, phenolic acids, alkaloids, phenolic acids, hydrolysable tannins and volatile oils that account for their medicinal properties (Shahwar *et al.*, 2012). *Conyza bonariensis* (Plate 1) is an annual plant and one of the most common species of genus Conyza used in traditional treatment of different types of ailment.

Plate 1: Appearance of *C. bonariensis* at the field site**.**

It is used in traditional treatment of sore throat, ringworm, chicken pox, bleeding from injuries, toothache diarrhea and constipation in Pakistan and China (Bauar, 1989; Thabit *et al.*, 2014; Shinwari *et al.*, 2015). Traditional healers in Rungwe district, Tanzania, use *C. bonariensis* to treat HIV/AIDS opportunistic infections (Kibonde *et al.*, 2018).

On the other hand, Zygophyllaceae is a plant family with about 240 species in 25 genera mostly distributed in tropical, subtropical and warm temperate areas (Dastagir *et al.*, 2012). *Tribulus terrestris* (Plate 2) is medicinally popular species in genus Tribulus. *Tribulus terrestris* possesses several secondary metabolites such as alkaloids, saponnins, tannins and glycosides responsible for its medicinal properties (Dastagir *et al.*, 2012). It is reported that, *T. terrestris* has high amount of steroidal saponnin which can inhibit *C. albicans* and *C. neoformans* growth (Hashimu *et al.*, 2014).

Although the traditional antimicrobial uses of *T. terrestris* are not widely reported, the antifungal and antibacterial activity of the plant's extracts have been reported (Al-Bayati and Al-Mola, 2008; Dastagir *et al.*, 2012; Hashimu *et al.*, 2012; Seyed *et al.*, 2017). Like some other medicinal plants, the antimicrobial effectiveness of *T. terrestris* is geographically determined.

Plate 2: Appearance of *T. terrestris* at the field site.

In Pakistan, the plant's extracts demonstrated no antimicrobial activity but showed antimicrobial activity in Iraq and Iran (Kianbakht and Jahaniani, 2003; Hashimu *et al.*, 2014). Leaves of the plant are used traditionally to treat gonorrhoea, inflammation, leprosy, skin diseases, ulitis, and general body weakness at Gujarat region in India (Ram *et al.*, 2015).

Rubiaceae is the fourth largest plant family comprising of about 13 143 species in 611 genera most with medical uses (Davis *et al.*, 2009; Aro *et al.*, 2015). *Rubia cordifolia* (Plate 3) and other members of Rubiaceae family have various pharmacologically important

phytochemicals such as saponnins, alkaloids, falvonoids, tannins and glycosides (Kannan *et al.*, 2009). In China and India, *R. cordifolia* is used for treatment of various skin infections and in wound healing (Uzun *et al.*, 2018).

Plate 3: Appearance of *R. cordifolia* at the field site.

Like other medicinal plants, *R. cordifolia* is rich in secondary metabolites which give them antimicrobial and antioxidant properties (Arif *et al*., 2009; Thabit *et al*., 2014). In Rwandese refugee camps in Ngara district in Tanzania, *R. cordifolia* was used to cure gonorrhea and syphilis (Ramathal and Ngassapa, 2001).

2.7 Synergistic effect of plant extracts

Traditional healers often mix different herbs when prescribing medicines for their patients in order to boost the efficacy of individual herb. For example, traditional healers at Korogwe district in Tanzania mix fresh roots of *Carica papaya* with fresh roots of *Ocimum suave* to treat vaginal candidiasis (Runyoro *et al.*, 2006). It is reported that some active compounds

from medicinal plants demonstrate more pharmacokinetic when in combination with other relevant compounds than when in isolation (Rates, 2001). A mixed juice from leaves of *Ageratum conyzoides* L. and *Cocculus hirsutus* containing Coumarin, friedelin, sitosterol, stigmasterol, alkaloids, and conyzorigun active compounds from both plants is used to cure diarrhea by Kanikaran tribe in Southern Western Ghats of India (Ayyanar and Ignacimuthu, 2005). Proper active plant compounds can be mixed to boost their microbial inhibitory effects as a result of antimicrobial synergistic effects.

2.8 Safety and toxicity of medicinal plants

It is generally regarded that traditional medicines especially from medicinal plants are safe to human health. This trust is the reason to why traditional medicines are not accompanied by strictly prescribed doses to patients but given in approximation measurement. About 27 toxins used to manufacture cosmetics are reported to come from medicinal plants (George, 2011). If not appropriately used, medicinal plants can cause fatalities or health complications in humans. Still traditional medicines are considered to have fewer side effects than synthetic medicines (George, 2011). There is a need to investigate the safety of the traditional medicines to communicate to the public for precautious measures.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Chemicals and organisms tested

Distilled water was collected from Arusha Technical College and Sea salt was prepared by evaporating water collected from the Indian Ocean, along the Dar es Salaam Coast. Brine Shrimps (*Artemia salina*) eggs were purchased from Aquaculture innovations (Grahamstown 6140, South Africa). Para-iodonitrotetrazolium chloride dye (INT) and dimethyl sulfoxide (DMSO) were purchased from Sigma® (Poole, Dorset, UK. Ketoconazole was purchased from S Kant Healthcare LTD, Gujarat, India and Ciprofloxacin tablets were bought from Micro Lab LTD, India. Chloroform, ethyl acetate and methanol were purchased from Avantor performance materials in India. Both nutrient (agar and broth), Sabouraud‘s dextrose (agar and broth) were purchased from Hi Media Laboratories Pvt Ltd (Mumbai-India).

Selection of microorganisms for testing the potency of medicinal plants depended on the availability during the study and the pathogenic representativeness of a microbe (Cos *et al.*, 2006). Two fungi, one methillin-resistant Gram-positive bacterium and four Gram-negative bacteria were experimented. *Cryptococcus neoformans* (clinical isolate), *Candida albicans* (ATCC 90028), *Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa* (ATCC 29953), *Salmonella typhi* (ATCC 6539), *Salmonella typhimurium* (ATCC 14023) and *Staphylococcus aureus* (ATCC29213) obtained from the Department of Microbiology, Muhimbili University of Health and Allied Sciences (MUHAS) except for *S. typhimurium* and *S. aureus* that were collected from Mount Meru Arusha Regional Hospital.

3.2 Plant materials preparation and extraction

Conyza bonariensis, Tribulus terrestris and *Rubia cordifolia* growing in Arusha, Tanzania were identified by Emanuel Mboya from Tropical Pesticides Research Institute (TPRI) and voucher specimens AMB501, AMB502 and AMB503, respectively are kept at TPRI. The leaf, stem and roots of *C. bonariensis, T. terrestris* and *R. cordifolia* were harvested while observing the sustainability of the plants. The collected plant parts were washed thoroughly with tape water, dried in shade and pulverized with electrical grinder to form fine powders. From each of the plant material, 350 g of the macerated powder was subjected to sequential extraction using chloroform, ethyl acetate and methanol using the standard extraction procedure. Solvents were removed through the vacuum using rotary evaporator. In order to

get aqueous extracts, another 350 g of plant material was soaked twice in distilled water for 24 hours and the filtrates were freeze-dried. All extracts were stored in the refrigerator at -20 ^{o}C until the time to conduct of conducting bioassay experiments.

3.3 Antibacterial assay

The efficacy of each plant extract was tested against five human pathogenic bacterial strains namely *E. coli, P. aeruginosa, S. typhi, S. typhimurium* and *S. aureus*. The assay involved the experimental materials and test procedure are outlined below.

3.3.1 Experimental materials

(i) 100 mg of each plant extract
(ii) 100 mg of plant extracts mixed in 1:1 ratio (for synergistic testing)
(iii) Bacterial strains
(iv) Ninety six (96)-well microtitre plates
(v) Micropipettes
(vi) Digital beam balance
(vii) Sabouraud's dextrose broth
(viii) *para*-iodonitrotetrazolium (INT) chloride dye
(ix) Dimethyl sulfoxide (DMSO) for dilution and negative control
(x) Ciprofloxacin antibiotic tablets for positive control

3.3.2 Test procedure

To determine the minimum inhibitory concentration (MIC) of the extracts against bacteria, Eloff (1998) micro dilution method was adopted with minor modifications. Antibacterial activity of individual extracts and synergistic effect of higher antibacterial activity plant parts were assessed. One hundred milligram (100 mg) of each extract measured by analytical balance was dissolved into 1 ml of DMSO to form 100 mg/mL stock solutions for unmixed extracts and test synergistic effect of extracts, 50 mg of the *C. bonariensis* leaf/root and 50 mg of *T. terrestris* leaf/root extracts were mixed in 1:1 to form 100 mg dissolved 1ml of DMSO to form 100 mg/mL stock solutions.

A 50 µL of Sabouraud's dextrose broth was loaded into sterilized 96-well microtitre plates followed by 50 µL of extracts in first well of each row to make a total volume of 100 µL. The plant extracts and Sabouraud's dextrose broth were thoroughly mixed in the first rows, 50 µL of the mixture from the first rows was shifted to the second rows, and shifting was repeated

down the columns until the last row where 50 µL were discarded. Ciprofloxacin tablets were used as positive control whereas DMSO and the row which contained only broth as growth control, represented negative control. Fifty microliter (50 µl) of bacterial suspensions (0.5 MacFarland) was added in each well and incubated at 37°C for 24 hours. Also, in each well, 20 µL of 0.02% p-iodonitrotetrazolium (INT) chloride dye was added and micro plates were incubated for 1-hour at 37 °C.

The change in the colour of INT was observed where formation of pink indicated live bacteria and persistence of the colour of INT meant the bacteria were dead as it is indicated in plate 4. The lowest concentration of the extracts that killed or did not allow the survival of the bacterium was described as Minimum Inhibitory Concentration (MIC), and thus the effective dose against the tested organism.

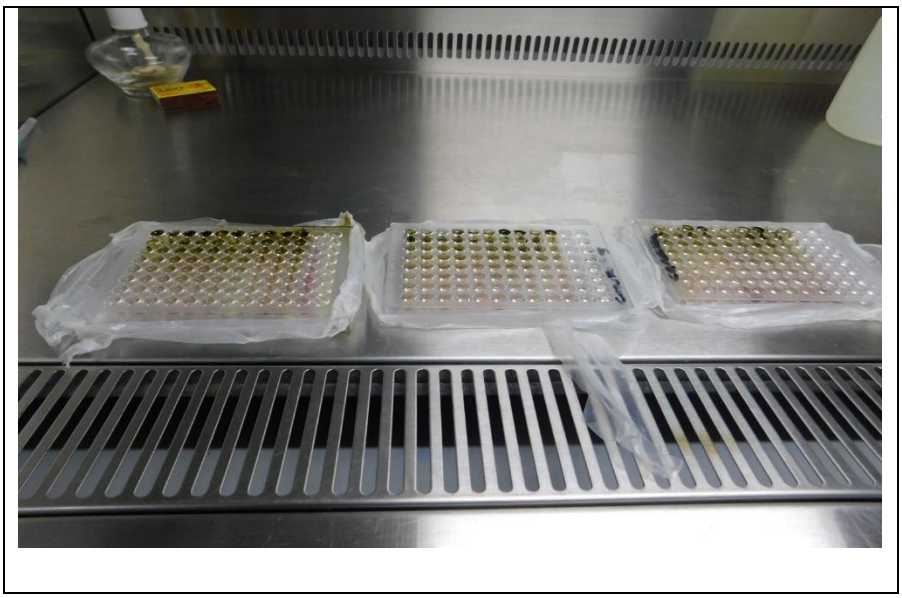

Plate 4: Test procedure for pathogenic microbes using 96 wells plates

3.4 Antifungal assay

The efficacy of each plant extract was tested against two human pathogenic fungal strains namely *C. albicans* and *C. neoformans*. The test materials and procedure used are outlined below.

3.4.1 Experimental materials

(i) 100 mg of each plant extract

(ii) 100 mg of plant extracts mixed in 1:1 ratio

(iii) *Candida albicans* and *C. neoformans* fungal strains

(iv) Ninety six (96)-well microtitre plates
(v) Micropipettes
(vi) Digital beam balance
(vii) Sabouraud's dextrose broth
(viii) *para*-iodonitrotetrazolium (INT) chloride dye
(ix) Dimethyl sulfoxide (DMSO) for dilution and negative control
(x) Ciprofloxacin antibiotic tablets for positive control

3.4.2 Test procedure

To determine the minimum inhibitory concentration (MIC) of the extracts against fungi, Eloff (1998) micro dilution method, with minor modifications, was used determine the minimum inhibitory concentration (MIC) of the extracts against fungi. To test antifungal activity the extracts, 100 mg of each extract was measured by analytical balance and dissolved into 1mL of DMSO to form 100 mg/mL stock solutions. Fifty microliter (50 µL) of Sabouraud's dextrose broth was loaded into sterilized 96-well microtitre plates followed by 50 µL of extracts in first well of each row to make a total volume of 100 µL.

The extracts and Sabouraud's dextrose broth were thoroughly mixed in the first rows, before 50 µL of the mixture from the first rows was shifted to the second rows, and shifting continued down the columns until the last row where 50 µL were discarded. Ketoconazole (100 µg/mL) was used as positive control whereas DMSO and the row which contained only broth as growth control, represented negative control. Fifty microliter (50 µL) of fungal (0.5 MacFarland) was added in each well and incubated at 37°C for 24 hours. Also, in each well, 20 µl of 0.02% *para*-iodonitrotetrazolium (INT) chloride dye was added and microplates were incubated for 1-hour at 37°C. Formation of pinkish colour from INT showed the presence of live bacteria and the wells which did not form pink colour contained dead fungus (Plate 5).

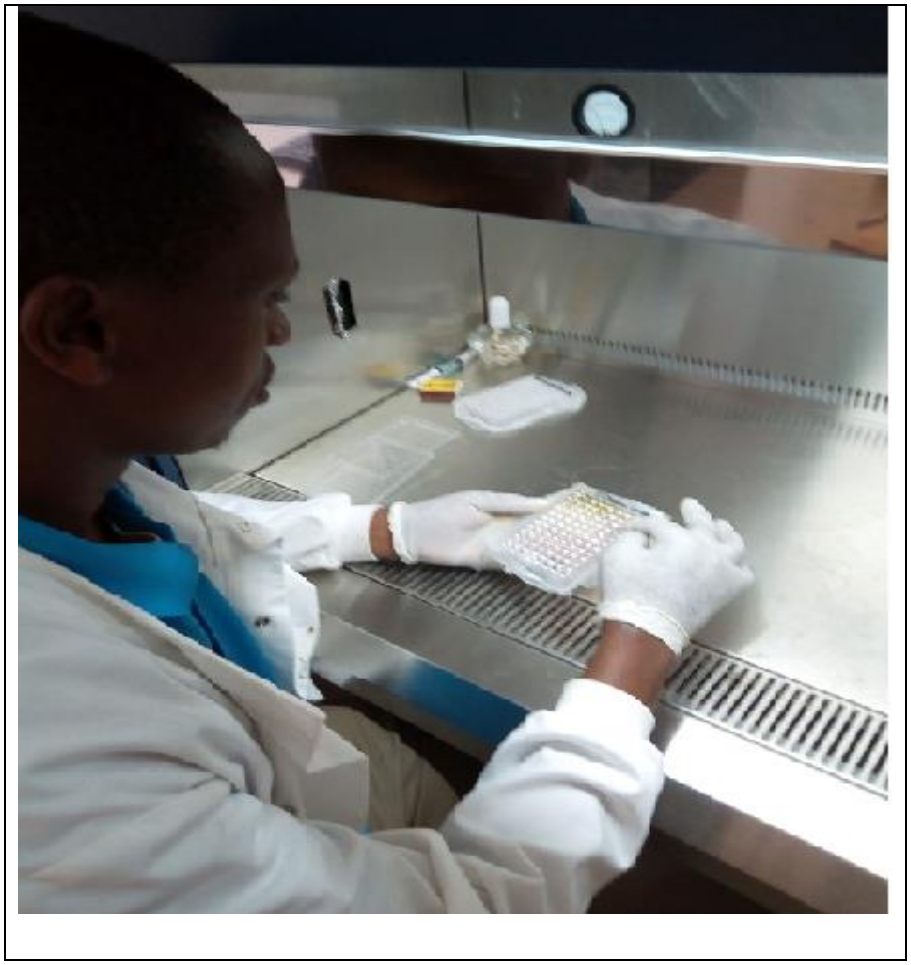

Plate 5: Recording MIC from microtitre plate inoculated with *C. albicans* and *C. neoformans* fungal strains

Even though 100% DMSO prohibits *C. albicans* growth, it has no effect in determining MIC because only the survival of the organism causes colour change (Kowero *et al*., 2016). Antifungal synergistic effect of the extracts was determined by combining 50 mg of *C. bonariensis* leaf/root extracts and 50 mg of *T. terrestris* leaf/root extracts in 1:1 to form 100 mg that was dissolved 1 ml of DMSO to form 100 mg/mL stock solutions. After mixing and making stock solutions, 50 µL of Sabouraud's dextrose broth was loaded into sterilized 96-well microtitre plates followed by 50 µl of extracts in first well of each row to make a total volume of 100 µl, then mixed followed by taking 50 µl of the mixture from the first rows to the second rows, and shifting continued down the columns until the last row where 50 µl was discarded.

The lowest concentration of the extracts that killed or did not allow the survival of fungus was described as minimum inhibitory concentration (MIC), the lowest effective dose against the tested organism.

3.5 Microbial statistical data analysis

Data were analyzed by using two-way analysis of variance (ANOVA) and differences among the means were determined for significance at P = 0.05 using statistical package for social science. All measurements were obtained in triplicate and expressed as mean value ± standard deviation of the mean.

3.6 Cytotoxicity test

Cytotoxicity tests are primary indicators of the cytotoxicity of plant extracts. In this study, cytotoxicity of *C. bonariensis*, *T. terrestris* and *R. cordifolia* extracts was tested against brine shrimp larvae. The materials and experimental protocol for cytotoxicity tests are outlined below.

3.6.1 Experimental materials

(i) Brine Shrimps
(ii) Sea salt
(iii) Dimethyl sulfoxide (DMSO)

3.6.2 Experimental protocol

The experiments were done according to Meyer *et al*. (1982) with some minor modifications.

(i) Media preparation

Artificial sea salt was prepared by dissolving 3.8 g of sea salt into one liter of distilled water. The artificial seawater was poured into a sterilized hatching container partitioned into two unequal parts, one part with brine shrimps eggs was covered with black paper to create dark while the second part was illuminated by light. Shrimp eggs (500 mg) were sprinkled into the covered part of the tank and a lamp illuminated on the uncovered part in order to attract the hatched shrimps. The mature nauplii were collected after between 24 and 36 hours of hatching. Brine shrimp larvae were used as indicator organisms for cytotoxicity assay (Meyer *et al.,* 1982).

(ii) Test procedure

Sample extracts were dissolved in dimethyl sulphoxide (DMSO) to make a stock solution of 40 mg/ml each, which were tested in duplicate at 240, 120, 80, 40, 24 and 8 µg/ml concentrations. In every tested concentration, 10 brine shrimp larvae were added including

positive and negative control of cyclophosphamide drug and DMSO, respectively (Meyer *et al., 1982*). The number of survivors was counted after 24 hours of incubation under the illumination condition.

(iii) Cytotoxicity statistical data analysis

The number of survived larvae was established and the LC_{50} values, regression equations together with regression factor (R_2) and Confidence intervals (95% CI) were obtained using Microsoft Excel analysis program.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 General results and discussion

The World Health Organization (WHO), estimated from one of its surveys that approximately 43% of deaths in developing countries are caused by infectious diseases. The pursuit for new effective, affordable, safe and accessible antimicrobial drugs to replace drugs compromised by microbial drug resistance is obligatory. Evaluation of traditional medicine has been vital in the process of developing new antimicrobial drugs (Muhammad *et al*., 2014). The antimicrobial and cytotoxicity of three traditionally used medicinal plants were studied and the results emanated from the study of *Conyza bonariensis, Tribulus terrestris* and *Rubia cordifolia* extracts are summarized in Table 1 with both positive and negative control results inclusive. *Para*-iodonitrotetrazolium chloride dye distinguished wells whose microbes have been inhibited or killed by compounds in the extracts from those with live microbes. Wells with live microbes turned pinkish while those with dead microbes did not turn pink. The stem bark of *R. cordifolia* were not considered in the evaluation because traditional healers and literature sparingly mention its medicinal use. Interpretation of MIC values was conducted as per Eloff (1998) that 0.05-0.5 mg/mL strong activity, 0.6-1.5 mg/mL moderate activity and above 1.5 mg/mL weak activity. All extracts showed moderate to weak antimicrobial potencies but at varying levels. The type of the solvent used in extraction impacted antimicrobial effectiveness of the plant. All solvents except water produced effective antimicrobial extracts to all tested microbes in effectiveness order ethyl acetate > chloroform > methanol > aqueous with average MIC 5.37, 7.01, 7.83 and 19.9, respectively.

Table 1: Minimum inhibitory concentration of plant extracts to the selected bacteria and fungi

Plant extracts	Minimum inhibition concentration (mg/mL)						
	Bacteria					Fungi	
	E. coli	*S. typhimurium*	*S. aureus*	*S. typhi*	*P. earuginosa*	*C. albicans*	*C. neoformans*
CBLA	25	12.5	12.5	25	12.5	25	25
CBLC	6.25	6.25	0.7813	3.125	6.25	3.125	3.125
CBLE	1.5625	3.125	1.5625	6.25	0.7813	1.5625	3.125
CBLM	3.125	1.5625	3.125	6.25	1.5625	12.5	3.125
CBSA	6.25	6.25	12.5	12.5	25	25	12.5
CBSC	6.25	3.125	6.25	1.5625	3.125	12.5	0.7813
CBSE	6.25	0.7813	6.25	6.25	3.125	12.5	6.25
CBSM	3.125	3.125	6.25	12.5	3.125	6.25	1.5625
CBRA	25	25	12.5	25	12.5	25	6.25
CBRC	1.5625	6.25	1.5625	3.125	3.125	6.25	1.5625
CBRE	6.25	1.5625	0.7813	0.7813	1.5625	1.5625	3.125
CBRM	6.25	3.125	1.5625	1.5625	3.125	12.5	3.125
TTLA	6.25	> 25	12.5	12.5	25	>25	12.5
TTLC	3.125	12.5	6.25	12.5	1.5625	12.5	6.25
TTLE	3.125	6.25	3.125	0.7813	3.125	6.25	1.5625
TTLM	12.5	12.5	6.25	12.5	6.25	6.25	12.5
TTSA	25	25	12.5	25	12.5	25	12.5
TTSC	6.25	25	6.25	1.5625	3.125	6.25	1.5625
TTSE	6.25	6.25	3.125	3.125	1.5625	6.25	6.25
TTSM	3.125	12.5	6.25	6.25	6.25	6.25	3.125
TTRA	> 25	> 25	12.5	25	25	> 25	25
TTRC	12.5	12.5	6.25	3.125	3.125	6.25	12.5
TTRE	6.25	3.125	6.25	6.25	0.7813	12.5	12.5
TTRM	3.125	6.25	1.5625	3.125	6.25	25	12.5
RCLA	>25	>25	>25	>25	>25	>25	25
RCLC	25	25	12.5	6.25	12.5	>25	12.5
RCLE	25	12.5	12.5	6.25	12.5	25	6.25
RCLM	12.5	25	12.5	25	12.5	25	12.5
RCRA	12.5	25	25	12.5	25	>25	25
RCRC	3.125	3.125	6.25	1.5625	3.125	6.25	3.125
RCRE	1.5625	6.25	3.125	3.125	3.125	3.125	1.5625
RCRM	3.125	12.5	6.25	6.25	6.25	6.25	3.125
Cipr	0.7813	0.3906	0.7813	0.3906	0.3906	N/A	N/A
Keto		N/A	N/A	N/A	N/A	0.7813	0.3906

Key: CBLA - *C. bonariensis* leaf aqueous extract, CBLC - *C. bonariensis* leaf chloroform extract, CBLE - *C. bonariensis* leaf ethyl acetate extract, CBLM - *C. bonariensis* leaf methanol extract, CBSA - *C. bonariensis* stem aqueous extract, CBSC - *C. bonariensis* stem chloroform extract, CBSE - *C. bonariensis* stem ethyl acetate extract, CBSM - *C. bonariensis* stem methanol extract, CBRA - *C. bonariensis* root aqueous extract, CBRC - *C. bonariensis* root chloroform extract, CBRE - *C. bonariensis* root ethyl acetate extract, CBRM - *C. bonariensis* root methanol extract, TTLA - *T. terrestris* leaf aqueous extract, TTLC - *T. terrestris* leaf chloroform extract, TTLE - *T. terrestris* leaf ethyl acetate extract, TTLM - *T. terrestris* leaf methanol extract, TTSA - *T. terrestris* stem aqueous extract, TTSC - *T. terrestris* stem chloroform extract, TTSE - *T. terrestris* stem ethyl acetate extract, TTSM - *T. terrestris* stem methanol extract, RCLA - *R. cordifolia* leaf aqueous extract, RCLC - *R. cordifolia* leaf chloroform extract, RCLE - *R. cordifolia*

leaf ethyl acetate extract, RCLM - *R. cordifolia* leaf methanol extract, RCRA - *R. cordifolia* root aqueous extract, RCRC - R.cordifolia root chloroform extract, RCRE - R. cordifolia root ethyl acetate extract, RCRM, *R. cordifolia* root methanol extract, Keto - Ketoconazole, Cipr – Ciprofloxacin, N/A-- Not applicable.

The average MIC for all extracts from each plant were compared and the antimicrobial effectiveness order C. *bonariensis* > *T. terrestris* > *R. cordifolia* was observed with their respective average MIC 7.49, 10.2 and 13.6. Results in Table 2 describe differential antimicrobial effectiveness between *C. bonariensis*, *T. terrestris* and *R. cordifolia* extracts.

Table 2: The plant mean comparison for antimicrobial effectiveness between *C. bonariensis* (CB), *T. terrestris* (TT) and *R. cordifolia* (RC)

Plant extract	Microbes (Mean ± Standard deviation)						
	Bacteria					Fungi	
	E. coli	*S. typhimurium*	*S.aureus*	*S. typhi*	*P.earuginosa*	*C.albicans*	*C.neoformans*
CBA	18.8 (10.8)	14.6 (9.5)	12.5 (-)	20.8(7.2)	16.7 (7.2)	25 (-)	14.6 (9.5)
CBC	4.7 (2.7)	5.2 (1.8)	2.9 (3.0)	2.6 (0.9)	4.2 (1.8)	7.3 (4.8)	1.8 (1.2)
CBE	4.7 (2.7)	1.8 (1.2)	2.9 (3.0)	4.4 (3.2)	1.8 (1.2)	5.2 (6.3)	4.2 (1.8)
CBM	4.2 (1.8)	2.6 (0.9)	3.6 (2.4)	6.8 (5.5)	2.6 (0.9)	10.4 (3.6)	2.6 (0.9)
T n=12	8.1 (8.1)	6.1 (6.7)	5.5 (4.7)	8.7 (8.6)	6.3(7.1)	11.9 (8.9)	5.8 (6.8)
P-value	0.039	0.045	0.003	0.006	0.004	0.002	0.042
Average mean				**7.49**			
TT A	18.8 (10.8)	25.0 (-)	12.5 (-)	20.8 (7.2)	20.8 (7.2)	25 (-)	16.7 (7.2)
TTC	7.3 (4.8)	16.7 (7.2)	6.3 (-)	5.7 (5.9)	2.6 (0.9)	8.3 (3.6)	6.8 (5.5)
TTE	5.2 (1.8)	5.2 (1.8)	4.2 (1.8)	3.4 (2.7)	1.8 (1.2)	8.3 (3.6)	6.8 (5.5)
TTM	6.3 (5.4)	14.4 (3.6)	4.7 (2.7)	7.3 (4.8)	6.3 (-)	12.5 (10.8)	9.4 (5.4)
T n=12	9.4 (8.0)	14.3 (8.5)	6.9 (3.7)	9.3 (8.5)	7.9 (8.6)	13.5 (8.8)	9.9 (6.6)
P-value	0.111	0.002	0.001	0.017	0.001	0.028	0.216
Average mean				**10.2**			
RCA	18.8 (8.8)	25.0	25.0 (-)	18.8 (8.8)	25.0 (-)	25 (-)	25 (-)
RCC	14.1 (15.5)	14 (15.5)	9.4 (4.4)	3.9 (3.3)	7.8 (6.6)	16 (13.3)	7.8 (6.6)
RCE	13.3 (16.6)	9.4 (4.4)	7.8 (6.6)	4.7 (2.2)	7.8 (6.6)	14 (15.5)	3.9 (3.3)
RCM	7.8 (6.6)	18.8 (8.8)	9.4 (4.4)	15.6 (13.3)	9.4 (4.4)	16 (13.3)	7.8 (6.6)
T n=8	13.5 (10.4)	16.8 (9.3)	12.9 (8.3)	10.7 (9.4)	12.5 (8.7)	18 (10.3)	11.1 (9.5)
P-value	0.856	0.460	0.053	0.305	0.072	0.800	0.043
Average mean				**13.6**			

Key: CBA-- *C. bonariensis* aqueous extract, CBC-- *C. bonariensis* chloroform extract, CBE-- *C. bonariensis* ethyl acetate extract, CBM-- *C. bonariensis* methanol extract, TTA—*T. terrestris* aqueous extract, TTC— *T.terrestris* chloroform extract, TTM— *T. terrestris* methanol extract, RCA— *R. cordifolia* aquoues extract, RCC— *R. cordifolia* chloroform extract, RCE— *R. cordifolia* ethyl acetate extract, RCM— *R. cordifolia* methanol extract.

Root extracts were statistically significant ($p = 0.05$) most effective followed by leaves and stem extracts. Table 3 depicts antimicrobial effectiveness among the plant parts. The results showed no individual extract which produced strong antibacterial activity between 0.05-0.5 mg/mL. However, about 14% (32) of all treatments (224) had moderate antibacterial activity between 0.7825 mg/mL and 1.5625 mg/mL. Two of thirty three treatments demonstrated moderate antifungal activity against *C. neoformans*. Mixing of plant extracts revealed antimicrobial synergistic effect and consequently lowered MIC. Cytotoxicity testing of plant extracts revealed *T. terrestris* chloroform, *R. cordifolia* aqueous and *C. bonariensis* methanol have mildly toxicity to brine shrimps of LC_{50} 52.5069, 75.8198 and 79.0076 µg/mL, respectively while the rest of extracts did not intoxicate brine shrimps by producing LC_{50} higher than 100 µg/mL.

Table 3: Mean comparison for antimicrobial effectiveness between leaf (L), root (R) and stem (S) of all plants

Plant part extract	Microbes (Mean ± Standard deviation)						
	Bacteria					Fungi	
	E.coli	*S. typhimurium*	*S. aureus*	*S. typhi*	*P. earuginosa*	*C. albicans*	*C. neoformans*
Leaf aqueous	18.8 (11)	20.8 (7.2)	16.7 (7.2)	20.8 (7.2)	20.8 (7.2)	25 (-)	20.8 (7.2)
Leaf chloroform	11.5 (12)	14.6 (9.5)	6.5 (5.9)	7.3 (4.8)	6.8 (5.5)	13.5 (11)	7.3 (4.8)
Leaf ethyl acetate	9.9 (13.1)	7.3 (4.8)	5.7 (5.9)	4.4 (3.2)	5.5 (6.2)	10.9 (12.4)	3.6 (2.4)
Leaf methanol	9.4 (5.4)	13 (11.7)	7.3 (4.8)	14.6 (9.5)	6.8 (5.5)	14.6 (9.5)	9.4 (5.4)
Total n=12	12.4 (9.9)	13.9 (9.0)	9.0 (6.9)	11.8 (8.8)	10.0 (8.4)	16 (9.9)	10.3 (8.1)
P-value	0.696	0.362	0.168	0.061	0.046	0.353	0.019
Root aqueous	20.8 (7.2)	25(-)	16.7(7.2)	20.8 (7.2)	20.8 (7.2)	25 (-)	18.8 (10.8)
Root chloroform	5.7 (5.9)	7.3 (4.8)	4.7 (2.7)	2.6 (0.9)	3.1 (-)	6.3 (-)	5.7 (5.9)
Root ethyl acetate	4.7 (2.7)	3.6 (2.4)	3.4 (2.7)	3.4 (2.7)	1.8 (1.2)	5.7 (5.9)	5.7 (5.9)
Root methanol	4.2 (1.8)	7.3 (4.8)	3.1 (2.7)	3.6 (2.4)	5.2 (1.8)	14.6 (9.5)	6.3 (5.4)
Total n=12	8.9 (8.4)	10.8 (9.2)	7.0 (6.9)	7.6 (8.7)	7.7 (8.6)	12.9 (9.5)	9.1 (8.6)
P-value	0.009	0.001	0.013	0.001	0.001	0.009	0.155
Stem aqueous	15.6 (13)	15.6 (15)	12.5 (-)	18.8 (8.8)	18.8 (8.8)	25 (-)	12.5 (-)
Stem chloroform	6.3 (-)	14.1 (16)	6.3 (-)	1.6 (-)	3.1 (-)	9.4 (4.4)	1.2 (0.6)
Stem ethyl acetate	6.3 (-)	3.5 (3.9)	4.7 (2.2)	4.7 (2.2)	2.3 (1.1)	9.4 (4.4)	6.3 (-)
Stem methanol	3.1 (-)	7.8 (6.6)	6.3 (-)	9.4 (4.4)	4.7 (2.2)	6.3 (-)	2.3 (1.1)
Total n=8	8.9 (7.1)	10.3 (9.7)	7.4 (3.3)	8.6 (7.9)	7.2 (8.0)	12.5 (8.2)	5.6 (4.8)
P-value	0.381	0.684	0.007	0.94	0.063	0.013	<0.001

4.2 Antibacterial activity

All extracts exhibited moderate to weak antibacterial activity to all tested bacteria. The lowest concentration to inhibit bacterial growth was 0.7825 mg/mL expressed by seven plant extracts to some bacteria (Table 1). It was found that, *C. bonariensis* extracts produced five (5) MIC values of 0.7825 mg/mL MIC against five (5) bacteria while only one (1) *T. terrestris* extract inhibited a bacterium at 0.7825 mg/mL MIC. All *R. cordifolia* extracts demonstrated weak antibacterial activity against the tested bacteria. Ethyl acetate extracts were most effective followed by chloroform extracts with the former demonstrating five of six moderate most effective concentrations (0.7825 mg/mL) against the tested bacteria. Aqueous and methanol formed weak inhibitory concentrations to all bacteria tested. Despite being popular among traditional healers, aqueous extracts demonstrated the least activity among all solvents. The differential in solubility of phytochemicals in the solvents enhances the composition and concentration phytochemicals and consequently affecting the antimicrobial effectiveness of the extracts.

Gram negative *S. typhimurium* with average mean 10.16 (6.25) was on average the most resistant bacterium (Table 4) whereas Gram-positive *S. aureus* bacterium was on average the most sensitive bacterium with MIC average mean 5.57 (4.59). Moreover, all tested bacteria except *E. coli* were sensitive to at least one extract at 0.7825 mg/mL. The best weak inhibitory concentration to *E. coli* was 1.5625 mg/mL from *C. bonariensis* leaf ethyl acetate, *C. bonariensis* root chloroform, and *R. cordifolia* root ethyl acetate. *Escherichia coli* was almost resistant to all *T. terrestris* and *R. cordifolia* extracts except *T. terrestris* root ethyl acetate by expressing MIC higher than 1.5625 mg/mL. *C. bonariensis* leaf and *C. bonariensis* root extracts inhibited *S. aureus* at 0.7825 mg/mL concentration while *C. bonariensis* root and *T. terrestris* root extracts at the same concentration inhibited *S. typhi*. Likewise *P. aeruginosa* was twice sensitive to 0.7825 mg/mL concentration from *C. bonariensis* leaf and *T. terrestris* root extracts but *S. typhimurium* was inhibited at 0.7825 mg/mL only by *C. bonariensis* stem extract. The stem extracts of *T. terrestris* and all extracts from *R. cordifolia* failed to inhibit bacterial growth at concentrations 0.7825 mg/mL. *Rubia cordifolia* leaf extracts were least effective registering MIC from 6.25 mg/mL and higher to the tested bacteria. The weak antibacterial activity of *R. cordifolia* and of *T. terrestris* stem does not suggest them to be considered for treatment of bacterial infections unless in combination with other medicines. Variations in sensitivity of bacteria to different plants extracts indicate either the bacteria have different resistance mechanism and/or the extracts have different

composition and concentration of antibacterial phytochemical compounds. All bacteria tested were resistant to *R. cordifolia* extracts apart from *E. coli* and *S. typhi* which were moderately vulnerable at chloroform and ethyl acetate root extracts. *Rubia cordifolia* leaf extracts showed low antibacterial activity with minimum inhibition of 6.25 mg/mL or higher. Seventy one percent (71.4%) of the 0.7825 mg/mL were from *C. bonariensis* extracts while 28.6% was from *T. terrestris* extracts. The sensitivity of the tested bacteria to extracts varied considerably and the variation is represented by averages of their means and standard deviations shown in Table 4. The strong antibacterial activity of *C. bonariensis* extracts are associated by its essential oils which are also reported to delay inflammations during bacterial infections (Souza *et al*., 2003). In Uganda and Tanzania, due to its believed antimicrobial activity, *C. bonariensis* is traditionally used to treat opportunistic in HIV/AIDS patients (Mugisha *et al*., 2014; Kibonde *et al.*, 2018). The results of this study support the traditional use of Tanzanian *C. bonariensis* in the treatment of microbial infections, and suggest its consideration in the formulation of antibiotics due to its promising antibacterial activity.

4.3 Antifungal activity of extracts

The antifungal properties of *C. bonariensis*, *T. terrestris* and *R. cordifolia* were assessed using MIC of their extracts against *C. albicans* and *C. neoformans* fungal strains. Results obtained from the antifungal evaluation of the extracts are presented in Table 1. The classification and interpretation of MIC values was done according to Eloff (1998) that 0.05-0.5 mg/mL strong activity, 0.6-1.5 mg/mL moderate activity and above 1.5 mg/mL weak activity.

All extracts did not show strong activity to both *C. albicans* and *C. neoformans* (Table 1). However, some extracts demonstrated moderate antifungal activity between 0.7825 mg/mL - 1.5625 mg/mL. Only *C. bonariensis* stem chloroform extract demonstrated the minimum inhibitory concentration of 0.7825 mg/mL against *C. neoformans.* The 1.5625 mg/mL was displayed by five extracts namely *C. bonariensis* stem methanol, *C. bonariensis* root chloroform, *T. terrestris* leaf ethyl acetate, *T. terrestris* stem chloroform and *R. cordifolia* root ethyl acetate against *C. neoformans.* To portray more resistance to antifungals, *C. albicans* was not inhibited by extract concentrations less than 1.5625 mg/mL. Only *C. bonariensis* leaf ethyl acetate and *C. bonariensis* root ethyl acetate (2/32) equal to 6 % of all extracts expressed moderate antifungal activity against *C. albicans* compared to 19% (6/33) of all extracts that produced moderate antifungal activity against *C. neoformans*.

However, *C. albicans* was more sensitive than *C. neoformans* to some extracts like *C. bonariensis* leaf ethyl acetate and *C. bonariensis* root ethyl acetate by showing a moderate sensitivity of 1.5625 mg/mL while *C. neoformans* demonstrated weak sensitivity above 1.5625 mg/mL to these extracts. The resistance of *C. albicans* to medicinal plant extracts had been reported by some researchers (Pappas *et al*., 2015). However, the Tanzanian *C. bonariensis* demonstrated some antifungal activity against *C. albicans* than the Turkish which demonstrated lower MIC against the same fungus (Ayaz *et al*., 2017).

Despite that leaves of *R. cordifolia* are used traditionally to treat fungal infections in Tanzania, their antifungal activities reported for the first time by this research to have shown weak antifungal activity. This work reports for the first time that both Tanzanian *C. bonariensis* and *T. terrestris* have demonstrated moderate and weak antifungal activities in Tanzania against *C. neoformans* and *C. albicans* fungal strains. Traditional healers can be advised to abandon the use of *R. cordifolia* in treatment of fungal and bacterial infections unless in combination with other medicines because its extracts have confirmed low antifungal activity. Nevertheless, *C. bonariensis* and *T. terrestris* which have demonstrated moderate antifungal activity can be used as traditional antifungal medicine but also can be considered in searching for antifungal drugs. The averages of MIC values for selected extracts namely CBLE, CBLM, TTRC, TTRE, TTRM, TTLC, TTLE and TTRM were determined as shown in Table 4.

Table 4: Summary characteristics of unmixed extracts from CBRC, CBRE, CBRM, CBLC, CBLE, CBLM, TTRC, TTRE, TTRM, TTLC, TTLE and TTM

Microbes	Sum	Mean	SD	Median
E. coli	146.88	9.18	8.48	6.25
S. typhimurium	162.50	10.16	8.33	6.25
S. aureus	89.06	5.57	4.59	4.69
S. typhi	146.88	9.18	8.78	6.25
P. aeruginosa	112.50	7.03	7.90	3.13
C. albicans	206.25	12.89	9.17	12.50
C. neoformans	143.75	8.98	7.62	6.25

4.4 Antibacterial synergistic effects

Extracts from different plants and plant parts have different secondary metabolites which can be combined to either form antimicrobial synergistic or antagonistic effect. In addition, the polarity of solvents affects composition and quantity during extraction of phytochemical

compounds. Since herbalists in Tanzania are usually prescribing herbal products prepared by mixing plant parts, extracts were mixed and evaluated for their antimicrobial activity and results are indicated in Table 5. The antimicrobial synergistic and antagonistic effects of the plant extracts have previously been reported by researchers (Adwan and Mhanna, 2008; Uzun *et al.*, 2018). In this study, antibacterial synergistic effect was demonstrated more than antagonistic effect. The minimum inhibitory concentrations of some mixed extracts revealed strong antibacterial activity to *E. coli* and *S. typhi* bacteria. *Escherichia coli* was sensitive at 0.3906 mg/mL of *C. bonariensis* leaf chloroform mixed with *CBLC-CBE* and *CBLC-CBRCL*. The extract CBLC had weak activity of 6.25 mg/mL against *E. coli* but MIC value was lowered to 1.5625 mg/mL when combined with CBRC against the same bacterium. A combination of CBLC and TTLC proved synergistic effect of phytochemicals by demonstrating a strong antibacterial activity of 0.3906 mg/mL against *S. typhi*. The extracts that had moderate or weak antibacterial activity have revealed strong activities on combination. Moderate antibacterial activity to each tested bacterium increased dramatically from unmixed to mixed extracts. Twenty four of forty one (24/41) mixed extracts displayed moderate antibacterial activity against *E. coli* which is equivalent to 58.5% and 56% (23/41) of extracts shown moderate activity against *P. aeruginosa*. Furthermore, 53.7% (22/41), 48.8% (20/41) and 46% (19/41) exhibited moderate antibacterial activity to *S. typhi*, *S. typhimurium* and *S. aureus*, respectively.

Table 5: Antimicrobial activities of mixed extracts from *C. bonariensis* and *T. terrestris* leaf and root extracts

Mixed plant extracts	Minimum inhibition concentration (mg/mL)						
	Bacteria					Fungi	
	E. coli	*S.typhimurium*	*S. aureus*	*S. typhi*	*P.earuginosa*	*C.albicans*	*C.neoformans*
CBLCCBLE	0.3906	3.125	1.5625	0.7813	1.5625	1.5625	1.5625
CBLCCBLM	1.5625	1.5625	6.25	1.5625	3.125	3.125	0.7813
CBLCCBRC	0.7813	6.25	1.5625	6.25	1.5625	6.25	3.125
CBLCCBRE	0.3906	1.5625	0.7813	3.125	3.125	3.125	1.5625
CBLCCBRM	1.5625	3.125	3.125	1.5625	6.25	1.5625	3.125
CBLECBRC	12.5	6.25	3.125	6.25	0.7813	0.7813	1.5625
CBLECBLM	3.125	6.25	12.5	1.5625	3.125	3.125	1.5625
CBLECBRE	3.125	0.7813	3.125	3.125	1.5625	1.5625	6.25
CBLECBRM	0.7813	1.5625	1.5625	1.5625	3.125	3.125	1.5625
CBLMCBRC	3.125	1.5625	3.125	0.7813	6.25	1.5625	1.5625
CBLMCBRE	6.25	1.5625	0.7813	0.7813	1.5625	6.25	1.5625
CBLMCBRM	6.25	3.125	1.5625	12.5	6.25	1.5625	6.25
CBLCTTLC	0.7813	6.25	1.5625	0.3906	3.125	3.125	3.125
CBLCTTLE	1.5625	6.25	6.25	1.5625	6.25	6.25	1.5625
CBLCTTLM	3.125	3.125	0.7813	1.5625	6.25	1.5625	1.5625
CBLETTLC	6.25	3.125	1.5625	0.7813	1.5625	0.7813	0.7813
CBLETTLE	0.7813	6.25	1.5625	3.125	0.7813	12.5	3.125
CBLETTLM	1.5625	0.7813	3.125	1.5625	0.7813	6.25	3.125
CBLMTTLC	6.25	0.7813	3.125	3.125	1.5625	3.125	1.5625
CBLMTTLE	1.5625	1.5625	6.25	1.5625	6.25	1.5625	3.125
CBLMTTLM	1.5625	6.25	1.5625	6.25	1.5625	6.25	1.5625
CBLCTTRC	3.125	1.5625	3.125	1.5625	1.5625	6.25	3.125
CBLCTTRE	3.125	3.125	0.7813	1.5625	3.125	1.5625	1.5625
CBLCTTRM	0.7813	1.5625	3.125	3.125	1.5625	6.25	0.7813
CBLETTRC	0.7813	1.5625	1.5625	0.7813	6.25	1.5625	3.125
CBLETTRE	1.5625	6.25	1.5625	1.5625	1.5625	6.25	6.25
CBLETTRM	6.25	3.125	0.7813	6.25	1.5625	1.5625	0.7813

Table 5: Antimicrobial activities of mixed extracts from *C. bonariensis* and *T. terrestris* leaf and root extracts (continuous)

Mixed plant extracts	Minimum inhibition concentration (mg/mL)						
	Bacteria					Fungi	
	E. coli	*S.typhimurium*	*S. aureus*	*S. typhi*	*P.earuginosa*	*C.albicans*	*C.neoformans*
CBLMTTRE	0.7813	0.7813	3.125	3.125	3.125	6.25	0.7813
CBLMTTRM	1.5625	6.25	0.7813	1.5625	1.5625	6.25	6.25
TTLCTTLE	0.7813	0.7813	3.125	6.25	3.125	3.125	6.25
TTLCTTLM	1.5625	1.5625	3.125	3.125	1.5625	12.5	3.25
TTLETTLM	1.5625	3.125	6.25	1.5625	1.5625	6.25	6.25
TTLETTRC	0.7813	1.5625	1.5625	1.5625	3.125	1.5625	3.125
TTLETTRE	1.5625	3.125	3.125	0.7813	0.7813	1.5625	1.5625
TTLETTRM	0.7813	3.125	0.7813	1.5625	1.5625	3.125	3.125
TTRCTTRE	0.7813	0.7813	1.5625	1.5625	0.7813	6.25	1.5625
TTRETTRM	1.5625	1.5625	3.125	3.125	1.5625	1.5625	3.125
TTLMTTRC	3.125	1.5625	6.25	3.125	3.125	1.5625	6.25
TTLMTTRE	1.5625	3.125	3.125	6.25	1.5625	3.125	3.125
TTLMTTRM	3.125	6.25	3.125	3.125	1.5625	6.25	3.125
Cipro	0.7813	0.3906	0.7813	0.3906	0.3906	N/A	N/A
Keto	N/A	N/A	N/A	N/A	N/A	0.7813	0.3906

Key: CBLCCBLE - *C. bonariensis* leaf chloroform extract and *C. bonariensis* leaf ethyl acetate extract, CBLCCBLM - *C. bonariensis* leaf chloroform extract and*C.bonariensis* leaf ethyl methanol extract, CBLCCBRC- *C. bonariensis* leaf chloroform extract and *C. bonariensis* root chloroform extract, CBLCCBRE- *C. bonariensis* leaf chloroform extract and *C. bonariensis* root ethyl acetate extract, CBLCCBRM- *C. bonariensis* leaf chloroform extract and *C. bonariensis* root methanol extract, CBLECBRC- *C. bonariensis* leaf ethyl acetate extract and *C. bonariensis* root chloroform extract, CBLECBLM- *C. bonariensis* leaf ethyl acetate extract and *C. bonariensis* leaf methanol extract, CBLECBRE- *C. bonariensis* leaf ethyl acetate extract and *C. bonariensis* root ethyl acetate extract, CBLECBRM- *C. bonariensis* leaf ethyl acetate extract and *C. bonariensis* root methanol extract, CBLMCBRC- *C. bonariensis* leaf methanol extract and *C. bonariensis* root chloroform extract, CBLMCBRE- *C. bonariensis* leaf methanol extract and *C. bonariensis* root ethyl acetate extract, CBLMCBRM- *C.bonariensis* leaf methanol extract and *C. bonariensis* root methanol extract, CBLCTTLC-*C. bonariensis* leaf chloroform extract and *T. terrestris* leaf chloroform extract, CBLCTTLE- *C. bonariensis* leaf chloroform extract and *T. terrestris* leaf ethyl acetate extract, CBLCTTLM- *C. bonariensis* leaf chloroform extract and *T. terrestris* leaf methanol extract, CBLETTLC- *C. bonariensis* leaf ethyl acetate extract and *T. terrestris* leaf chloroform extract, CBLETTLE- *C. bonariensis* leaf ethyl acetate extract and *T. terrestris* leaf ethyl acetate extract, CBLETTLM- *C. bonariensis* leaf ethyl acetate extract and *T. terrestris* leaf methanol extract, CBLMTTLC- *C. bonariensis* leaf methanol

extract and *T. terrestris* leaf chloroform extract, CBLMTTLE- *C. bonariensis* leaf methanol extract and *T. terrestris* leaf ethyl acetate extract, CBLMTTLM- *C.bonariensis* leaf methanol extract and*T.terrestris* leaf methanol extract, CBLCTTRC- *C. bonariensis* leaf chloroform extract and *T. terrestris* root chloroform extract, CBLCTTRE- *C. bonariensis* leaf chloroform extract and*T. terrestris* root ethyl acetate extract, CBLCTTRM- *C.bonariensis* leaf chloroform extract and *T. terrestris* root methanol extract, CBLETTRC- *C. bonariensis* leaf ethyl acetate extract and *T. terrestris* root chloroform extract, CBLETTRE- *C. bonariensis* leaf ethyl acetate extract and *T. terrestris* root ethyl acetate extract, CBLETTRM- *C. bonariensis* leaf ethyl acetate extract and *T. terrestris* root methanol extract, CBLMTTRC- *C. bonariensis* leaf methanol extract and *T. terrestris* root chloroform extract, CBLMTTRE- *C. bonariensis* leaf methanol extract and *T. terrestris* root ethyl acetate extract, CBLMTTRM- *C. bonariensis* leaf methanol extract and *T. terrestris* root methanol extract, TTLCTTLE-*T. terrestris* leaf chloroform extract and *T. terrestris* leaf ethyl acetate extract, TTLCTTLM- *T. terrestris* leaf chloroform extract and *T. terrestris* leaf methanol extract, TTLETTLM- *T. terrestris* leaf ethyl acetate extract and *T. terrestris* leaf methanol extract, TTLETTRC- *T. terrestris* leaf ethyl acetate extract and *T. terrestris* root chloroform extract, TTLETTRE- *T. terrestris* leaf ethyl acetate extract and *T. terrestris* root ethyl acetate extract, TTLETTRM- *T. terrestris* leaf ethyl acetate extract and *T. terrestris* root methanol extract, TTRCTTRE- *T. terrestris* root chloroform extract and *T. terrestris* root ethyl acetate extract, TTRETTRM- *T. terrestris* root ethyl acetate extract and *T. terrestris* root methanol extract, TTLMTTRC- *T. terrestris* leaf methanol extract and *T. terrestris* root chloroform extract, TTLMTTRE- *T. terrestris* leaf methanol extract and *T. terrestris* root ethyl acetate extract, TTLMTTRM- *T. terrestris* leaf methanol extract and *T. terrestris* root methanol extract.

The sequence of bacterial sensitivity to extracts in mixed extracts changed with *S. aureus*, a Gram-positive bacteria that was most sensitive before extracts were mixed becoming most resistant after mixing of the extracts. This might be due to the nature of phytochemicals present in the extracts and the structural differences between Gram-positive and Gram-negative bacteria. The sensitivity sequence of tested bacteria to extracts after computation of the average means changed from *S. aureus* > *P. earuginosa* > *E. coli* > *S. typhi* > *S. typhimurium* before mixing of extracts to *S. aureus* > *S. typhi* > *E. coli* > *S. typhimurium* > *P. earuginosa* after extracts were mixed (Table 6).

Table 6: Summary characteristics of mixed extracts from CBRC, CBRE, CBRM, CBLC, CBLE, CBLM, TTRC, TTRE, TTRM, TTLC, TTLE and TTM

Microbes	Sum	Mean	SD	Median
E. coli	86.72	2.89	2.64	1.56
S. typhimurium	96.09	3.20	2.17	3.13
S. aureus	81.25	2.71	2.43	1.56
S. typhi	85.55	2.85	2.62	1.56
P. earuginosa	98.44	3.28	2.10	3.13
C. albicans	112.50	3.75	2.70	3.13
C. neoformans	74.22	2.47	1.72	1.56

The general increase in sensitivity of bacteria in mixed than in unmixed extracts validates antimicrobial synergistic effects of phytochemicals present in plant extracts. The means of MIC values of extracts without synergistic effect (Table 4) and the means of MIC values of extracts with synergistic effect were compared as depicted in Fig. 1. Microbes were susceptible to mixed extracts than they were to unmixed extracts and the means of MIC values were low in mixed compared to unmixed.

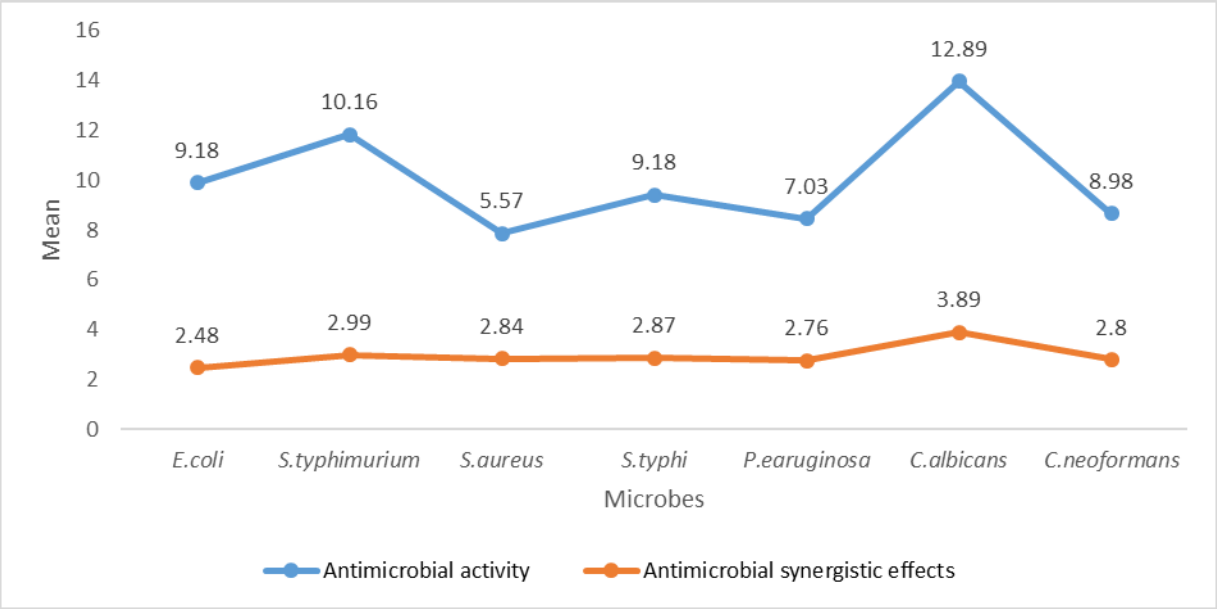

Figure 1: Mean comparison between extracts without synergistic and those with synergistic effect.

4.5 Antifungal synergistic effects

After the extracts showed less promising antifungal activities against *C. albicans* and *C. neoformans*, some extracts were mixed to test for antifungal synergistic effect of phytochemicals present in plant extracts. The results obtained from mixed plant extracts exposed better antifungal potencies of the plants by revealing higher vulnerability of the fungal strains in mixed extracts as presented in Table 5. Seventeen extracts (41.5%) and twenty extracts (48.8%) out of forty one showed moderate antifungal activity against *C. albicans* and *C. neoformans*, respectively. However, no any extract in both mixed and unmixed which exhibited strong antifungal activity from this research and the best MIC shown in both mixed and unmixed extracts was 0.7825 mg/mL. It was registered once in unmixed extracts by *C. bonariensis* stem chloroform extract against *C. neoformans*. In mixed extracts, the value was established by a combination of *C. bonariensis* leaf ethyl acetate with *C. bonariensis* root chloroform and that of *C. bonariensis* leaf ethyl acetate with *T. terrestris* leaf chloroform against *C. albicans*. In unmixed extracts, *C. bonariensis* leaf ethyl acetate and *C. bonariensis* root chloroform had inhibited *C. albicans* at moderate concentration of 1.5625 mg/mL and *T. terrestris* leaf chloroform inhibited the same fungus at 3.125 mg/mL. Only *C. bonariensis* stem chloroform extract was able to inhibit the growth of *C. neoformans* at was 0.7825 mg/mL extract concentration. The same fungus, *C. neoformans* experienced

growth inhibition from five mixtures of extracts at 0.7825 mg/mL namely *C. bonariensis* leaf chloroform mixed with *C. bonariensis* leaf methanol extract, *C. bonariensis* leaf ethyl acetate mixed with *T. terrestris* leaf chloroform. Others were *C. bonariensis* leaf chloroform mixed with *T. terrestris* root methanol, *C. bonariensis* leaf ethyl acetate with *T. terrestris* root methanol and *C. bonariensis* leaf methanol extract mixed with *T. terrestris* root ethyl acetate. The occurrence of antifungal synergistic effect is confirmed by the fact that all extracts showed higher antifungal activities before they were mixed than after they had been mixed. Previous studies have reported the antimicrobial synergistic effect of *C. bonariensis* phytochemicals and synthetic antibiotics like mpicillin, cephalothin, chloramphenicol and tetracycline (Silva and Fernandes, 2010). Furthermore, Uzun *et al*. (2018) unveil the antimicrobial synergistic effect which occurs among different plant extracts. Different active compounds are target different drug receptor sites of the pathogens and inhibit the pathogen to quickly adapt the multi active component drugs.

4.6 Cytotoxicity results and discussion

Cytotoxicity test on Brine shrimps larvae (*Artemia salina*) is a simple and effective guide for active cytotoxic agents. Plant extracts may have cytotoxic effect because they may contain compounds which interfere with the normal cell cycle activities. Two treatments were done for every plant extract and results are shown in Table 7. The survival of the larvae did not show a considerable variation between two treatments done for each concentration.

Table 7: Raw cytotoxicity data

Conc.	Number of survivors/Sample code											
(µg/ml)	CBA	CBC	CBE	CBM	TTA	TTC	TTE	TTM	RCA	RCC	RCE	RCM
240	0, 0	4, 6	0, 0	0, 0	9, 10	0, 0	3, 5	9, 7	1, 1	8, 7	10, 10	5, 8
120	6, 6	5, 6	5, 4	2, 2	10, 10	0, 1	5, 5	9, 10	2, 3	9, 9	10, 10	9, 10
80	9, 9	9, 9	8, 9	7, 6	10, 10	2, 3	8, 9	10, 10	5, 4	10, 10	10, 10	10, 10
40	10, 10	10, 10	10, 9	8, 8	10, 10	7, 5	10, 10	10, 10	8, 8	10, 10	10, 10	10, 10
24	10, 10	10, 10	10, 10	10, 10		8, 10	10, 10	10, 10	9, 10			10, 10
8		10, 10	10, 10	10, 10		3, 5	10, 10					10, 10

The classification of the toxicity findings was L_{50} < 1.0µg/mL— highly toxic; 1-10 µg/mL— toxic; 10.0-30.0 µg/mL— moderately toxic; > 30 < 100 µg/mL— mildly toxic and >100 µg/mL as non-toxic (Meyer *et al*., 1982). *Tribulus terrestris* chloroform, *R. cordifolia*

aqueous and *C. bonariensis* methanol had mildly toxicity of LC_{50} 52.5069, 75.8198 and 79.0076 µg/mL, respectively while other extracts showed negative toxicity with LC_{50} higher than 100 µg/ml (Table 8). Despite the plants showing little toxicity, in vivo research is required to further and justify these findings. Since brine shrimps cytotoxicity experiments are preliminary tests, in vivo testing of the extracts is required to determine the toxicity effects on human being.

Table 8: Analyzed cytotoxicity data

SAMPLE CODE	LC_{50} (µg/ml)	95% CI; (µg/ml) Lower Limit – Upper Limit.	REGRESSION EQUATION	RENTETION FACTOR (R^2)
CBA	122.1161	119.6026-124.6824	Y=130.34logx-221.99	0.8923
CBC	212.1390	150.5610-298.9022	Y = 70.712logx-114.52	0.8538
CBE	104.3163	84.0265-129.5053	Y = 100.26logx- 152.36	0.868
CBM	79.0076	64.0585-97.4452	Y = 103.39logx-146.2	0.9454
TTA	*	*	*	*
TTC	52.5069	41.7077-66.1022	Y=94.18logx-112.01	0.9321
TTE	164.7666	123.0403-220.6434	Y= 83.027logx-134.06	0.9072
TTM	>1000	#	Y= 42.763logx-82.357	0.983
RCA	75.8198	59.6729-96.3358	Y=90.553logx-120.22	0.9792
RCC	718.4012	419.5734-1229.3724	Y=52.112logx-98.851	0.9988
RCE	*	*	*	*
RCM	>1000	#	Y=76.177logx-148.22	0.943
Cyclophosphamide	16.365	12.006 - 22.305	Y = 69.9680logx - 34.9360	0.994929

KEY: LC_{50} = Lethal Concentration, CI = Confidence Interval, * =Not active up maximum concentration of 240 µg/ml, CBA—*C. bonariensis* aqueous, CBC—*C. bonariensis* chloroform, CBE — *C. bonariensis* ethyl acetate, CBM — *C. bonariensis* methanol, TTA — *T. terrestris* aqueous, TTC — *T. terrestris* chloroform, TTE — *T. terrestris* ethyl acetate, TTM — *T. terrestris* methanol, RCA — *R. cordifolia* aqueous, RCC — *R. cordifolia* chloroform, RCE — *R. cordifolia* ethyl acetate, RCM — *R. cordifolia* methanol.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

This study has established that Tanzanian *C. bonariensis*, *T. terrestris* and roots of *R. cordifolia* possess moderate antimicrobial properties. The results from this study support the use leaves, roots and the stem barks of *C. bonariensis* and *T. terrestris* as well as roots of *R. cordifolia* for curing fungal and bacterial infections since they inhibited moderate and strong antimicrobial effect. Combination of extracts coupled the synergistic effect of phytochemical compounds. Leaves of *R. cordifolia* showed very weak antimicrobial activity with the lowest MIC being 6.25 mg/mL against *S. typhi* bacterium and *C. neoformans* fungus. The tested plants are safe for medical application since they they established low cytotoxicity. The moderate antimicrobial potency and the low toxicity of the tested plants accrue the traditional usability of *C. bonariensis*, *T. terrestris* and *R. cordifolia* to treat microbial infections especially where accessibility and affordability of the synthetic modern medicine is not ease.

Since pathogens develop resistance to drugs as an evolutionary process that humans cannot prevent, then it is appealing to search for new drug precursors and drugs. Follow-up studies on the characterization, isolation and quantification of the active compounds from the studied plants is recommended. Further toxicity tests are also recommended. The survey on medicinal plants is required for conservation and commercialization

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RESEARCH OUTPUT

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In Vitro Antimicrobial Activity of *Conyza bonariensis* and *Tribulus terrestris* Growing in Tanzania

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Abstract

Objective: The aim of this study was to evaluate antimicrobial activity of *Conyza bonariensis* and *Tribulus terrestris* growing in Tanzania.

Background statement: The improvement of antimicrobial agents has protected and improved human life from fatal diseases since their discovery. The increase in microbial drug resistance demands for new effective drugs of which medicinal plants are a promising source.

Methods: Minimum inhibitory concentration (MIC) in 96-well micro dilution was used to determine antimicrobial activity. The method involved loading of 50 µL of Sabouraud's dextrose broth in each well. Then, 50 µL of plant extract fetched from 100 mg/mL stock solution to form 100 µL. Thereafter, 50 µL of the mixture from the first rows was repeatedly down the columns until the last row where 50 µL were discarded. Subsequently, 50 µl of microbial suspensions was added in each well and incubated at 37°C for 24 hours. In each well, 20 µL of 0.02% Para-iodonitrotetrazolium chloride dye (INT) was added to micro plates in order to distinguish between the wells with live microbes from those with dead microbes.

Results: All extracts demonstrated antimicrobial activity to tested bacterial and fungal strains. Seven extracts namely *C. bonariensis* leaf chloroform, *C. bonariensis* leaf ethyl acetate, *C. bonariensis* stem chloroform, *C. bonariensis* stem ethyl acetate, *C. bonariensis* root ethyl acetate, *T. terrestris* leaf ethyl acetate and *T. terrestris* root ethyl acetate inhibited *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Salmonella typhi* and *Cryptococcus neoformans* at 0.78125 mg/mL. Plant extracts demonstrated synergistic effect of their chemical components against the tested microbes. The strongest bactericidal and fungicidal of combined extracts was exhibited on *E. coli* and *C. neoformans* respectively. The extracts from *C. albicans* and *T. terrestris* revealed antimicrobial activity against seven tested microbes.

Conclusion: It was concluded that *C. bonariensis* and *T. terrestris* can be considered as possible sources of antimicrobial drug leads upon further phytochemical investigations.

Keywords: Antimicrobial; *Conyza bonariensis*; *Tribulus terrestris*; Extract; MIC; Synergistic effect; Ethno-medical information

Introduction

Humans have used antibiotics to manage bacterial and fungal infections for the last seven decades [1]. Infectious diseases killed 3.25 million of children worldwide in the year 2013 [2] and bacterial infections accounted for 17.8% global human death [3]. The situation is worse in developing countries that may have been attributed by poor medication and little or non-affordability of proper antimicrobial drug. The prevalence of infectious diseases is high despite the availability of conventional antimicrobial drugs mainly because some microbes have acquired resistance to the present drugs [4].

In these circumstances, the search for effective, safe and affordable antimicrobial drugs is an appealing need. Ethno-medical information has gained popularity as one of reliable sources for drug templates [4]. It is estimated that about 25% of conventional drugs originate from medicinal plants [5].

It is further reported that about 56% of people in Kilimanjaro region use traditional medicines in their primary health care [6]. Tanzania is estimated to have about 1,000 medicinal plant species [7]. Despite the fact that the country is blessed with high diversity of medicinal plants, only few of them have been evaluated for their antimicrobial activity.

Conyza bonariensis is an annual herb present all over the world except in Antarctica [8,9]. It is used for treatment of ailment such as sore throat, ringworm, chicken pox, bleeding from injuries, toothache diarrhea and constipation in Pakistan and China [10,11]. In Tanzania, *C. bonariensis* is used for treatment of HIV/AIDS opportunistic infections [12]. *Tribulus terrestris* which is commonly known as spine/puncher vine is distributed in tropical, subtropical and warm temperate areas

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[13]. Leaves of the *T. terrestris* are used traditionally to treat gonorrhea, inflammation, leprosy, skin diseases, ulitis, and general body weakness at Gujarat region in India [14]. Traditionally, this herb is used to enhance libido, stimulate spermatogenesis, vermifuge and medicine to skin diseases [12,15].

Fungal and bacterial infections continue to cost the wellbeing of humans because, some pathogenic bacteria and fungi have acquired adaptive mechanisms to present antibacterial and antifungal drugs respectively [16]. Furthermore, the problem continues to cost peoples' life because of insufficient efforts to search for new and effective antimicrobial drugs. Although currently medicinal plants have been regarded as promising source of antimicrobial drugs, most of them have not been scientifically evaluated to be considered in drug production [17].

In searching for new antimicrobial drugs, *in vitro* evaluation of unstudied medicinal plants is essential at initial stages. That is why this study reports the antifungal and antibacterial activity on *C. bonariensis* and *T. terrestris* growing in Tanzania on four Gram-negative bacteria which are *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Salmonella typhimurium*, one Gram-positive Methelin-resistant bacterium *Staphylococcus aureus* as well as two fungal strains namely *Cryptococcus neoformans* and *Candida albicans*.

Materials and Methods

Acquisition of materials

Distilled water was collected from Arusha Technical College while seawater from Indian Ocean along the Dar es Salaam coast. Para-iodonitrotetrazolium chloride dye (INT) and dimethyl sulfoxide (DMSO) were purchased from Sigma and Alrich, St Louis, USA. Ketoconazole was purchased from S Kant Healthcare LTD, Gujarat, India and Ciprofloxacin tablets were bought from Micro Lab LTD, India. Chloroform, ethyl acetate and methanol were purchased from Avantor performance materials in India. Both nutrient (agar and broth), Sabouraud's dextrose (agar and broth) were purchased from Hi Media Laboratories Pvt Ltd (Mumbai-India). Selection of microorganisms for testing the potency of medicinal plants depended on the availability during the study on their pathogenic representativeness as suggested by Cos et al. [18]. The test microorganisms were two fungal strains namely *Cryptococcus neoformans* (clinical isolate) and *Candida albicans* (ATCC 90028), one Gram-positive bacterium *Staphylococcus aureus* (ATCC29213) and four Gram-negative bacteria namely *Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa* (ATCC 29953), *Salmonella typhi* (ATCC 6539) and *Salmonella typhimurium* (ATCC 14023) were obtained from the Department of Microbiology, Muhimbili University of Health and Allied Sciences (MUHAS).

Sample collection

Leaves, stem bark and roots of *C. bonariensis* and *T. terrestris* were collected from Nambala, Themi and Kaloleni areas in Arusha. Plant species were identified by Mr. Mboya from Tropical Pesticides Research Institute (TPRI) and voucher specimens AMB501 and AMB502 were are assigned for *C. bonariensis* and *T. terrestris* respectively. The leaves, stem barks and roots of *C. bonariensis* and *T. terrestris* were harvested while observing the sustainability of the plants.

Extraction process

The collected plant materials were washed thoroughly with tape water, dried in shade and pulverized with electrical grinder to form fine powders to increase surface area for extraction. From each of the plant material, 350 g of the powder was sequentially macerated twice by being completely immersed in 99.8% chloroform, 99.5% ethyl acetate and 99.5% methanol solvents for 48 hours at room temperature. All extracts were stored in the refrigerator at -20°C until the time of conducting bioassay experiments.

Antimicrobial assays

Minimum inhibitory concentration (MIC) of the extracts against bacteria and fungi was determined through micro dilution method using 96-well plates as proposed by Eloff [19] with minor modifications. Firstly, 100 mg of each plant extract was dissolved in 1 ml of DMSO to form 100 mg/mL stock solutions. In testing extracts' synergistic effect, 50 mg of one plant extract and the same amount of the other extract were mixed in a 1:1 ratio and then dissolved into 1 ml of DMSO to form 100 mg/mL stock solutions.

A 50 µL of Sabouraud's dextrose broth was loaded into sterilized 96-well microtitre plates followed by addition of 50 µl of 100 mg/mL extract in first well of each row to make a total volume of 100 µl in each of the first-row wells. The plant extracts and Sabouraud's dextrose broth were thoroughly mixed in the first rows. After that, 50 µl of the mixture from the first rows was shifted to the second rows and shifting continued down the columns until the last row where 50 µl from each well was discarded.

Next, 50 µl of microbial suspensions (0.5 MacFarland) were added in each well and incubated at 37°C for 24 hours followed by 20 µl of 0.02% p-iodonitrotetrazolium (INT) chloride dye and incubated for 1-hour at 37°C. Ketoconazole (100 µg/mL) and Ciprofloxacin were used as positive control for fungal and bacterial tests respectively. Dimethyl sulfoxide (DMSO) and the row which contained only broth were used as growth control representing negative control.

The change in the color of INT was observed where formation of pink indicated live microbes and persistence of the color of INT meant the microbes were dead. Even though 100% DMSO prohibits *C. albicans* growth, it has no effect in determining MIC because INT only causes color change to living organisms [20]. The lowest concentration of the extracts that killed or did not allow the survival of microbes was described as Minimum Inhibitory Concentration (MIC) that is the lowest effective dose against the tested organism.

Results

Results emanated from antimicrobial evaluation of *Conyza bonariensis* and *Tribulus terrestris* extracts are summarized in **Table 1**. The extracts were tested against four Gram-negative and one Gram-positive bacteria and two fungal strains and all the microbes are pathogenic to human being. The minimum inhibitory concentration (MIC) shows the activity of the extract against the microbes as presented in **Tables 1 and 2**. Eighty percent of the tested bacteria and 50% of the tested fungi were inhibited by the extracts at the MIC value of 0.78125 mg/mL.

Table 1 Antimicrobial activities of *C. bonariensis* and *T. terrestris* (leaf, stem and root bark).

Plant Extracts	Minimum Inhibition Concentration (mg/mL)							
	E. coli	*S. typhimurium*	*S. aureus*	*S. typhi*	*P. aeruginosa*	*C. albicans*	*C. neoformans*	S.E
CLC	6.25	6.25	0.78125	3.125	6.25	3.125	3.125	0.8125
CLE	1.5625	3.125	1.5625	6.25	0.78125	1.5625	3.125	0.697
CLM	3.125	1.5625	3.125	6.25	1.5625	12.5	3.125	1.4637
CSC	6.25	3.125	6.25	1.5625	3.125	12.5	0.78125	1.5609
CSE	6.25	0.78125	6.25	6.25	3.125	12.5	6.25	1.3623
CSM	3.125	3.125	6.25	12.5	3.125	6.25	1.5625	1.394
CRC	1.5625	6.25	1.5625	3.125	3.125	6.25	1.5625	0.7944
CRE	6.25	1.5625	0.78125	0.78125	1.5625	1.5625	3.125	0.7319
CRM	6.25	3.125	1.5625	1.5625	3.125	12.5	3.125	1.4637
TLC	3.125	12.5	6.25	12.5	1.5625	12.5	6.25	1.7717
TLE	3.125	6.25	3.125	0.78125	3.125	6.25	1.5625	0.797
TLM	12.5	12.5	6.25	12.5	6.25	6.25	12.5	1.2627
TSC	6.25	25	6.25	1.5625	3.125	6.25	1.5625	3.0849
TSE	6.25	6.25	3.125	3.125	1.5625	6.25	6.25	0.7624
TSM	3.125	12.5	6.25	6.25	6.25	6.25	3.125	1.1811
TRC	12.5	12.5	6.25	3.125	3.125	6.25	12.5	1.6504
TRE	6.25	3.125	6.25	6.25	0.78125	12.5	12.5	1.6592
TRM	3.125	6.25	1.5625	3.125	6.25	25	12.5	3.101
Cipr	0.78125	0.390625	0.78125	0.39062	0.390625	N/A	N/A	--
Keto		N/A	N/A	N/A	N/A	0.78125	0.390625	--

Key: CLC-*C. bonariensis* leaf chloroform, CLE-*C. bonariensis* leaf ethyl acetate, CLM-*C. bonariensis* leaf methanol, CSC-*C. bonariensis* stem chloroform, CSE-*C. bonariensis* stem ethyl acetate, CSM-*C. bonariensis* stem methanol, CRC-*C. bonariensis* root chloroform, CBRE-*C. bonariensis* root ethyl acetate, CRM-*C. bonariensis* root methanol, TLC-*T. terrestris* leaf chloroform, TLE-*T. terrestris* leaf ethyl acetate, TLM-*T. terrestris* leaf methanol, TSC-*T. terrestris* stem chloroform, TSE-*T. terrestris* stem ethyl acetate, TSM-*T. terrestris* stem methanol, Keto-Ketoconazole, Cipr-Ciprofloxacin, S.E-Standard error.

According to Rios and Recio [21], plant extracts that are suggested in the drug discovery initiatives are the ones with MIC values less than 1 mg/mL. In this study, *C. bonariensis* leaf chloroform (CLC), *C. bonariensis* leaf ethyl acetate (CLE), *C. bonariensis* stem chloroform (CSC), *C. bonariensis* stem ethyl acetate (CSE), *C. bonariensis* root ethyl acetate (CRE), *T. terrestris* leaf ethyl acetate (TLE) and *T. terrestris* root ethyl acetate (TRE) met that criterion. They exhibited MIC value of 0.78125 mg/mL against *S. aureus*, *P. aeruginosa*, *C. neoformans*, *S. typhimurium* and *S. typhi* (**Table 1**).

Table 2 Antimicrobial activities of mixed extracts from *C. bonariensis* and *T. terrestris* (leaf and root extracts).

Combined Plant Extracts	Minimum inhibition concentration (mg/mL)							
	E. coli	*S. typhimurium*	*S. aureus*	*S. typhi*	*P. aeruginosa*	*C. albicans*	*C. neoformans*	S. E
CLCCLE	0.390625	3.125	1.5625	0.78125	1.5625	1.5625	1.5625	0.3238

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CLCCLM	1.5625	1.5625	6.25	1.5625	3.125	3.125	0.78125	0.697
CLCCRC	0.78125	6.25	1.5625	6.25	1.5625	6.25	3.125	0.9448
CLCCRE	0.390625	1.5625	0.7812	3.125	3.125	3.125	1.5625	0.4429
CLCCRM	1.5625	3.125	3.125	1.5625	6.25	1.5625	3.125	0.6313
CLECRC	12.5	6.25	3.125	6.25	0.78125	0.78125	1.5625	0.16057
CLECLM	3.125	6.25	12.5	1.5625	3.125	3.125	1.5625	1.464
CLECRE	3.125	0.78125	3.125	3.125	1.5625	1.5625	6.25	0.6789
CLECRM	0.78125	1.5625	1.5625	1.5625	3.125	3.125	1.5625	0.3348
CLMCRC	3.125	1.5625	3.125	0.78125	6.25	1.5625	1.5625	0.697
CLMCRE	6.25	1.5625	0.78125	0.78125	1.5625	6.25	1.5625	0.9315
CLMCRM	6.25	3.125	1.5625	12.5	6.25	1.5625	6.25	1.441
CLCTLC	0.78125	6.25	1.5625	0.39062	3.125	3.125	3.125	0.7466
CLCTLE	1.5625	6.25	6.25	1.5625	6.25	6.25	1.5625	0.947
CLCTLM	3.125	3.125	0.7812	1.5625	6.25	1.5625	1.5625	0.697
CLETLC	6.25	3.125	1.5625	0.78125	1.5625	0.78125	0.78125	0.757
CLETLE	0.7812	6.25	1.5625	3.125	0.78125	12.5	3.125	1.585
CLETLM	1.5625	0.78125	3.125	1.5625	0.78125	6.25	3.125	0.7319
CLMTLC	6.25	0.78125	3.125	3.125	1.5625	3.125	1.5625	0.6789
CLMTLE	1.5625	1.5625	6.25	1.5625	6.25	1.5625	3.125	0.8352
CLMTLM	1.5625	6.25	1.5625	6.25	1.5625	6.25	1.5625	0.947
CLCTRC	3.125	1.5625	3.125	1.5625	1.5625	6.25	3.125	0.6313
CLCTRE	3.125	3.125	0.7812	1.5625	3.125	1.5625	1.5625	0.3702
CLCTRM	0.78125	1.5625	3.125	3.125	1.5625	6.25	0.78125	0.7319
CLETRC	0.7812	1.5625	1.5625	0.78125	6.25	1.5625	3.125	0.7319
CLETRE	1.5625	6.25	1.5625	1.5625	1.5625	6.25	6.25	0.947
CLETRM	6.25	3.125	0.7812	6.25	1.5625	1.5625	0.78125	0.9135
CLMTRC	3.125	0.78125	1.5625	6.25	6.25	1.5625	1.5625	0.8764
CLMTRE	0.78125	0.78125	3.125	3.125	3.125	6.25	0.78125	0.757
CLMTRM	1.5625	6.25	0.7812	1.5625	1.5625	6.25	6.25	0.992
TLCTLE	0.78125	0.78125	3.125	6.25	3.125	3.125	6.25	0.8475
TLCTLM	1.5625	1.5625	3.125	3.125	1.5625	12.5	3.25	1.4794
TLETLM	1.5625	3.125	6.25	1.5625	1.5625	6.25	6.25	0.8929
TLETRC	0.78125	1.5625	1.5625	1.5625	3.125	1.5625	3.125	0.3348
TLETRE	1.5625	3.125	3.125	0.78125	0.78125	1.5625	1.5625	0.3702
TLETRM	0.78125	3.125	0.7812	1.5625	1.5625	3.125	3.125	0.4126
TRCTRE	0.78125	0.78125	1.5625	1.5625	0.78125	6.25	1.5625	0.7403
TRETRM	1.5625	1.5625	3.125	3.125	1.5625	1.5625	3.125	0.3157
TLMTRC	3.125	1.5625	6.25	3.125	3.125	1.5625	6.25	0.7403
TLMTRE	1.5625	3.125	3.125	6.25	1.5625	3.125	3.125	0.5906

TLMTRM	3.125	6.25	3.125	3.125	1.5625	6.25	3.125	0.6696
Cipro	0.78125	0.390625	0.78125	0.39062	0.39063	N/A	N/A	
Keto	N/A	N/A	N/A	N/A	N/A	0.78125	0.390625	

Key: CLCCLE-*C. bonariensis* leaf chloroform and *C. bonariensis* leaf ethyl acetate, CLCCLM-*C. bonariensis* leaf chloroform and *C. bonariensis* leaf ethyl methanol, CLCCRC-*C. bonariensis* leaf chloroform and *C. bonariensis* root chloroform, CLCCRE-*C. bonariensis* leaf chloroform and *C. bonariensis* root ethyl acetate, CLCCRM-*C. bonariensis* leaf chloroform and *C. bonariensis* root methanol, CLECRC-*C. bonariensis* leaf ethyl acetate and *C. bonariensis* root chloroform, CLECLM-*C. bonariensis* leaf ethyl acetate and *C. bonariensis* leaf methanol, CLECRE-*C. bonariensis* leaf ethyl acetate and *C. bonariensis* root ethyl acetate, CLECRM-*C. bonariensis* leaf ethyl acetate and *C. bonariensis* root methanol, CLMCRC-*C. bonariensis* leaf methanol and *C. bonariensis* root chloroform, CLMCRE-*C. bonariensis* leaf methanol and *C. bonariensis* root ethyl acetate, CLMCRM-*C. bonariensis* leaf methanol and *C. bonariensis* root methanol, CLCTLC-*C. bonariensis* leaf chloroform and *T. terrestris* leaf chloroform, CLCTLE-*C. bonariensis* leaf chloroform and *T. terrestris* leaf ethyl acetate, CLCTLM-*C. bonariensis* leaf chloroform and *T. terrestris* leaf methanol, CLETLC-*C. bonariensis* leaf ethyl acetate and *T. terrestris* leaf chloroform, CLETLE-*C. bonariensis* leaf ethyl acetate and *T. terrestris* leaf ethyl acetate, CLETLM-*C. bonariensis* leaf ethyl acetate and *T. terrestris* leaf methanol, CLMTLC-*C. bonariensis* leaf methanol and *T. terrestris* leaf chloroform, CLMTLE-*C. bonariensis* leaf methanol and *T. terrestris* leaf ethyl acetate, CLMTLM-*C. bonariensis* leaf methanol and *T. terrestris* leaf methanol, CLCTRC-*C. bonariensis* leaf chloroform and *T. terrestris* root chloroform, CLCTRE-*C. bonariensis* leaf chloroform and *T. terrestris* root ethyl acetate, CLCTRM-*C. bonariensis* leaf chloroform and *T. terrestris* root methanol, CLETRC-*C. bonariensis* leaf ethyl acetate and *T. terrestris* root chloroform, CLETRE-*C. bonariensis* leaf ethyl acetate and *T. terrestris* root ethyl acetate, CLETRM-*C. bonariensis* leaf ethyl acetate and *T. terrestris* root methanol, CLMTRC-*C. bonariensis* leaf methanol and *T. terrestris* root chloroform, CLMTRE-*C. bonariensis* leaf methanol and *T. terrestris* root ethyl acetate, CLMTRM-*C. bonariensis* leaf methanol and *T. terrestris* root methanol, TLCTLE-*T. terrestris* leaf chloroform and *T. terrestris* leaf ethyl acetate, TLCTLM-*T. terrestris* leaf chloroform and *T. terrestris* leaf methanol, TLETLM-*T. terrestris* leaf ethyl acetate and *T. terrestris* leaf methanol, TLETRC-*T. terrestris* leaf ethyl acetate and *T. terrestris* root chloroform, TLETRE-*T. terrestris* leaf ethyl acetate and *T. terrestris* root ethyl acetate, TLETRM-*T. terrestris* leaf ethyl acetate and *T. terrestris* root methanol, TRCTRE-*T. terrestris* root chloroform and *T. terrestris* root ethyl acetate, TRETRM-*T. terrestris* root ethyl acetate and *T. terrestris* root methanol, TLMTRC-*T. terrestris* leaf methanol and *T. terrestris* root chloroform, TLMTRE-*T. terrestris* leaf methanol and *T. terrestris* root ethyl acetate, TLMTRM-*T. terrestris* leaf methanol and *T. terrestris* root methanol, S.E-Standard error.

Apparently, *C. albicans* and *E. coli* were the least susceptible to the tested extracts in which none of the extracts exhibited their growth at MIC value of 0.78125 mg/mL. Two extracts, namely CLE and CRE inhibited *C. albicans* at MIC value 1.5625 mg/mL while CLE and *C. bonariensis* root chloroform (CRC) inhibited *E. coli* at the same concentration.

It was noted that, 75% of extracts with MIC values below 1 mg/mL emanated from *C. bonariensis* extracts and 25% from *T. terrestris* extracts. This means *C. bonariensis* is preferably a more promising source for antimicrobial agents than *T. terrestris*. The lethality of phytochemicals contained in extracts was specific to the tested pathogens. The type of solvent used in extraction appeared to influence antimicrobial activity of the extracts. In a total of seven 0.78125 mg/mL MIC value, five of them were ethyl acetate extracts, two chloroform extracts and none methanol extracts.

Some phytochemicals demonstrate more pharmacokinetic when in combination with other relevant compounds than when in isolation [5].

In light of that, the synergistic effect of phytochemicals from different plant extracts were examined. The findings of the investigation demonstrated the presence of antimicrobial synergistic effect in the combined extracts which was evidenced by increasing antimicrobial efficacy of extracts. Microbial susceptibility to phytochemicals in combined extracts was determined to be species specific. *Escherichia coli* that was the most resistant bacterium in uncombined extracts became the most susceptible to mixed extracts. This was attributed by the observation that 32% of extracts' combinations were effective against *E. coli* at MIC values of 0.78125 mg/mL and 0.390625 mg/mL. The strongest synergistic effect came from CLC (6.25 mg/mL)-CLE (1.5625 mg/mL) and CLC (6.25 mg/mL)-CRE (6.25 mg/mL) combinations which inhibited the growth of *E. coli* at MIC values of 0.390625 mg/mL. Combinations CLC (06.25 mg/mL)-CRC (1.5625 mg/mL); CLE (1.5625 mg/mL)-CRM (6.25 mg/mL); CLE (1.5625 mg/mL)-CRM (6.25 mg/mL); CLC (6.25 mg/mL)-TLC (3.125 mg/mL); CLE (1.5625 mg/mL)-TLE (3.125 mg/mL); CLC (6.25 mg/mL)-TRM (3.125 mg/mL) and CLE (1.5625 mg/mL)-TRC (12.5 mg/mL) inhibited the growth of *E. coli* at MIC value 0.7825 mg/mL. Other combinations which inhibited the growth of *E. coli* at 0.7825 mg/mL MIC value to attest antimicrobial synergism of their phytochemicals were CLM (3.125 mg/mL)-TRE (6.25 mg/mL); TLC (3.125 mg/mL)-TLE (3.125 mg/mL); TLE (3.125 mg/mL)-TRC (12.5 mg/mL); TLE (3.125 mg/mL)-TRM (3.125 mg/mL); and TRC (12.5 mg/mL)-TRE (6.25 mg/mL).

Extract combinations of CLE (3.125 mg/mL)-CRE (1.5625 mg/mL); CLE (3.125 mg/mL)-TLM (12.5 mg/mL); CLM (1.5625 mg/mL)-TLC (12.5 mg/mL); CLM (1.5625 mg/mL)-TRC (12.5 mg/mL); CLM (1.5625 mg/mL)-TRE (3.125 mg/mL); TLC (12.5 mg/mL)-TLE (6.25 mg/mL) and TRC (12.5 mg/mL)-TRE (3.125 mg/mL) had strong antibacterial activity of 0.7825 mg/mL values against *S. typhimurium*.

Seven percent of the 41 extract combinations namely CLE (1.5625 mg/mL)-TRM (1.5625 mg/mL); CLM (3.125 mg/mL)-TRM (1.5625 mg/mL) and TLE (3.125 mg/mL)-TRM (1.5625 mg/mL) demonstrated antibacterial synergistic effect against *S. aureus* which was revealed lowered MIC values to 0.78125 mg/m. For the case of *S. typhi*, 4 out of 41 extract combinations had lower MIC values than before they were mixed. These combinations were (6.25 mg/mL)-CRC (3.125 mg/mL); CLE (6.25 mg/mL)-TLC (6.25 mg/mL) and CLE (6.25 mg/mL)-TRC (6.25 mg/mL) to 0.78125 mg/mL. However, extract combination CLC (3.125 mg/mL)-TLC (6.25 mg/mL) produced the strongest synergistic effect against *S. typhi* that lowed the MIC values to 0.390625 mg/mL. Extracts did not show remarkable synergistic effect against *Pseudomonas aeruginosa* bacterium.

The fungus *C. neoformans* was more susceptible to phytochemical synergistic effect than *C. albicans*. The extracts' combinations CLE (1.5625 mg/mL)-CRC (6.25 mg/mL) and CLE (6.25 mg/mL)-TLE (6.25 mg/mL) showed strong antifungal activity to *C. albicans* at MIC value of 0.78125 mg/mL while

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combinations CLC (3.125 mg/mL) CLM (3.125 mg/mL); CLE (3.125 mg/mL)-TLC (6.25 mg/mL); CLC (3.125 mg/mL)-TRM (12.5 mg/mL); CLE (3.125 mg/mL)-TRM (12.5 mg/mL); and CLM (3.125 mg/mL)-TRM (12.5 mg/mL) had strong antifungal activity to *C. neoformans* at MIC value of 0.78125 mg/mL.

Discussion

Evaluation of traditional medicine has been a reliable approach in drug discovery process [8]. However, the medicinal efficacy of some traditionally used medicinal plants have not been validated. In light of that fact, this study is reporting for the first time the antibacterial and antifungal activity of *C. bonariensis* and *T. terrestris* growing in Arusha region of Tanzania. According to Eloff [19], extracts with MIC values 0.05-0.5 mg/mL, 0.6-1.5 mg/mL and above 1.5 mg/mL represented strong, moderate and weak antimicrobial activity respectively. According to Rios and Recio [21], plant extracts which should be considered in drug discovery enterprises are the ones with MIC values less than 1 mg/mL. Extracts which displayed antifungal and antibacterial activity with MIC values 0.78125 and 0.39065 mg/mL were qualified for further examination as foundations of drug leads.

In this aspect, *C. bonariensis* leaf chloroform, *C. bonariensis* stem ethyl acetate, *C. bonariensis* root ethyl acetate, *C. bonariensis* leaf ethyl acetate, *T terrestris* leaf ethyl acetate and *T. terrestris* root ethyl acetate demonstrated antibacterial against *S. aureus*, *S. typhimurium*, *S. typhi* and *P. aeruginosa* with MIC of 0.78125 mg/mL are possible antibiotic templates for the diseases caused by these bacteria. Similarly, *C. bonariensis* stem chloroform extract inhibited *C. neoformans* with MIC of 0.78125 mg/mL qualify to be a possible antifungal drug lead for treatment of *C. neoformans* caused infections.

According to Uzun et al., [22] antimicrobial synergistic effect of phytochemicals occurs when different extracts are combined. Phytochemicals in *C. bonariensis* extracts produced synergistic effect against tested bacteria when combined with synthetic antibiotics [23]. The findings showed that *E. coli* was selectively more prone to combined extracts than rest of tested bacteria. The bacteria was resistant to all extracts at MIC values below 1 mg/mL. However, the bacterium was sensitive to about 32% of extracts' combinations. The fungus, *C. albicans* which initially was not inhibited by extracts before they were mixed, became susceptible to CBLE-CBRC and CBLE-TTLC extract combinations at MIC value of 0.78125 mg/mL. The effect of antimicrobial synergism of phytochemicals suggest that formulations of antimicrobial drugs should involve antimicrobial agents from different sources and extracted from different solvents. For better extraction, the type of solvent used, method of extraction and specifics during collection of materials have effects on results of antimicrobial activity of the extracts and should be valued [21].

The antimicrobial activity of *C. bonariensis* has been investigated in different parts of the world. *Conyza bonariensis* which is used to manage HIV/AIDS opportunistic infections in Tanzania and Uganda [24,25] possess antimicrobial activity. In Pakistan, the methanol and ethyl acetate extracts failed to inhibit the growth of *C. albicans*, *E. coli*, *S. aureus* and *S. typhimurium* at the MIC of 20 mg/mL [26]. However, the same study revealed that methanolic and ethyl acetate extracts from *C. bonariensis* inhibited *P. aeruginosa* and *Staphylococcus epidermidis* at 13.3 mg/mL and 16 mg/mL respectively [26]. Another study in Yemen discovered the antibacterial activity of *C. bonariensis* ethanol extracts against *S. aureus*, *E. coli* and *S. typhimurium* bacteria whereas *S. aureus* was mostly inhibited by extracts like in this study [11]. Furthermore, essential oil from Kenyan *C. bonariensis* exhibited antibacterial activity against two Gram-negative bacterial strains namely *E. coli* and *S. typhi* [27]. They found that, *S. typhi* was also more vulnerable than *E. coli* to *C. bonariensis* extracts. Although not very much studied, the antimicrobial activity of *T. terrestris* have been reported [15]. According to Hashim et al. [15], *T. terrestris* contain high amount of spirostanol saponins which inhibited fungal growth of *C. albicans* and *C. neoformans*. The antimicrobial activity of *T. terrestris* extracts are also in line with reported antifungal and antibacterial activity in Iran [28]. However, unlike the findings from this study, the Pakistani *T. terrestris* showed no antibacterial activity against *P. aeruginosa* bacterium [29]. The ethno-medical uses of *C. bonariensis* and *T. terrestris* in management of bacterial and fungal infections are endorsed by the findings of this study. The medicinal value of *T. terrestris* is highly associated with its high content of saponins which its composition varies due to geographical locations [15].

Conclusion

The extracts from *C. bonariensis* and *T. terrestris* revealed antifungal activity to two fungal strains namely *C. neoformans* and *C. albicans*. The extracts from the same plants also revealed antibacterial activities against four tested Gram-negative bacteria namely *E. coli*, *S. typhimurium*, *S. typhi* and *P. aeruginosa* and against one Methicillin- resistant Gram-positive bacterium namely *S. aureus*.

This study has publicized the antifungal and antibacterial activity of *C. bonariensis* and *T. terrestris* growing in Tanzania. It has further revealed the influence of phytochemicals' synergistic effect in management of bacterial and fungal infections.

Conflict of Interest

Authors declare that there is no competing interests exist.

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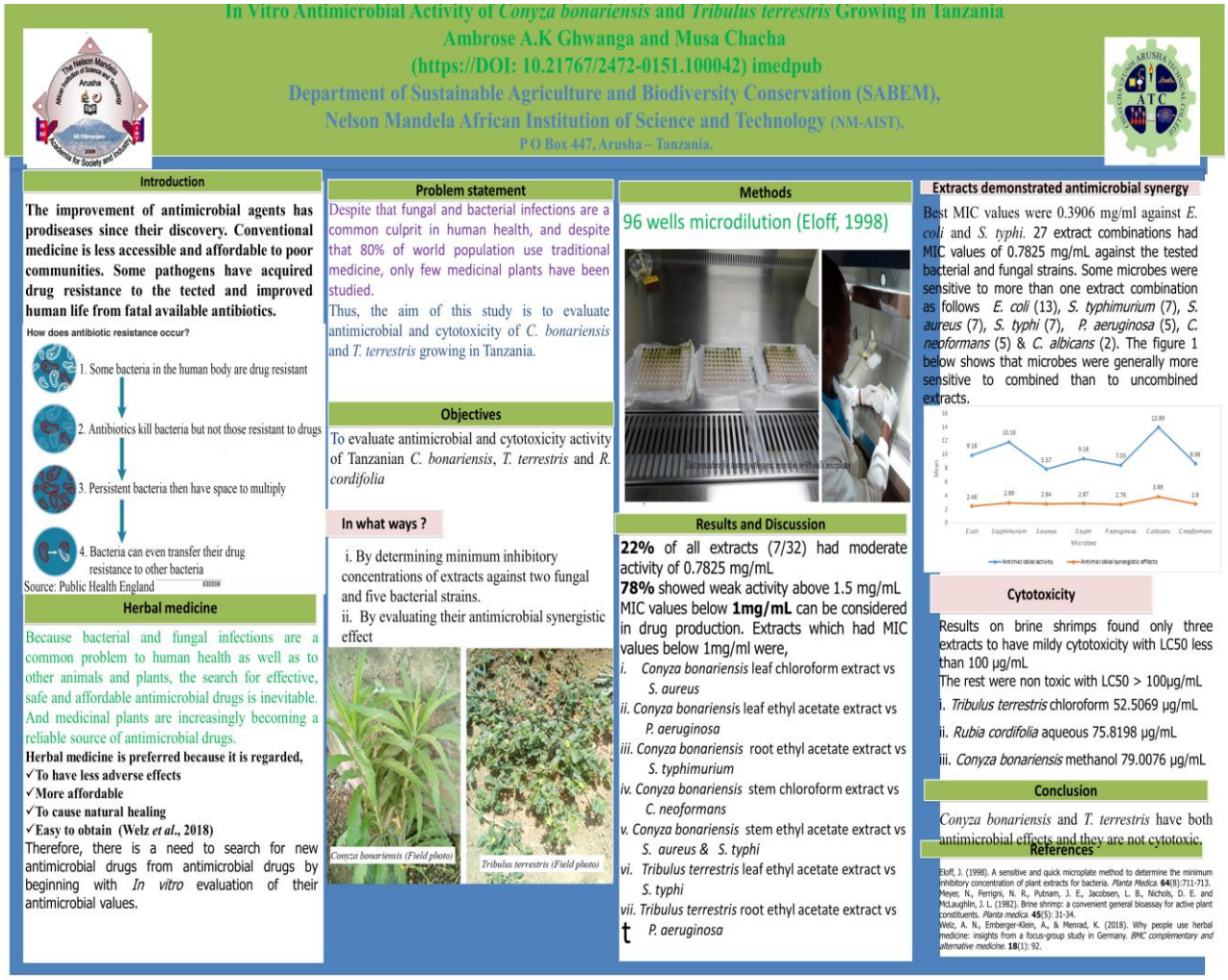

In Vitro Antimicrobial Activity of *Conyza bonariensis* and *Tribulus terrestris* Growing in Tanzania

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Introduction

The improvement of antimicrobial agents has prodiseases since their discovery. Conventional medicine is less accessible and affordable to poor communities. Some pathogens have acquired drug resistance to the tected and improved human life from fatal available antibiotics.

How does antibiotic resistance occur?

1. Some bacteria in the human body are drug resistant
2. Antibiotics kill bacteria but not those resistant to drugs
3. Persistent bacteria then have space to multiply
4. Bacteria can even transfer their drug resistance to other bacteria

Source: Public Health England

Herbal medicine

Because bacterial and fungal infections are a common problem to human health as well as to other animals and plants, the search for effective, safe and affordable antimicrobial drugs is inevitable. And medicinal plants are increasingly becoming a reliable source of antimicrobial drugs.

Herbal medicine is preferred because it is regarded,

- ✓**To have less adverse effects**
- ✓**More affordable**
- ✓**To cause natural healing**
- ✓**Easy to obtain (Welz *et al*., 2018)**

Therefore, there is a need to search for new antimicrobial drugs from antimicrobial drugs by beginning with *In vitro* evaluation of their antimicrobial values.

Problem statement

Despite that fungal and bacterial infections are a common culprit in human health, and despite that 80% of world population use traditional medicine, only few medicinal plants have been studied.

Thus, the aim of this study is to evaluate antimicrobial and cytotoxicity of *C. bonariensis* and *T. terrestris* growing in Tanzania.

Objectives

To evaluate antimicrobial and cytotoxicity activity of Tanzanian *C. bonariensis*, *T. terrestris* and *R. cordifolia*

In what ways ?

i. By determining minimum inhibitory concentrations of extracts against two fungal and five bacterial strains.

ii. By evaluating their antimicrobial synergistic effect

Conyza bonariensis (Field photo)

Tribulus terrestris (Field photo)

Methods

96 wells microdilution (Eloff, 1998)

Results and Discussion

22% of all extracts (7/32) had moderate activity of 0.7825 mg/mL

78% showed weak activity above 1.5 mg/mL

MIC values below **1mg/mL** can be considered in drug production. Extracts which had MIC values below 1mg/ml were,

i. *Conyza bonariensis* leaf chloroform extract vs *S. aureus*

ii. *Conyza bonariensis* leaf ethyl acetate extract vs *P. aeruginosa*

iii. *Conyza bonariensis* root ethyl acetate extract vs *S. typhimurium*

iv. *Conyza bonariensis* stem chloroform extract vs *C. neoformans*

v. *Conyza bonariensis* stem ethyl acetate extract vs *S. aureus* & *S. typhi*

vi. *Tribulus terrestris* leaf ethyl acetate extract vs *S. typhi*

vii. *Tribulus terrestris* root ethyl acetate extract vs *P. aeruginosa*

Extracts demonstrated antimicrobial synergy

Best MIC values were 0.3906 mg/ml against *E. coli* and *S. typhi*. 27 extract combinations had MIC values of 0.7825 mg/mL against the tested bacterial and fungal strains. Some microbes were sensitive to more than one extract combination as follows *E. coli* (13), *S. typhimurium* (7), *S. aureus* (7), *S. typhi* (7), *P. aeruginosa* (5), *C. neoformans* (5) & *C. albicans* (2). The figure 1 below shows that microbes were generally more sensitive to combined than to uncombined extracts.

Cytotoxicity

Results on brine shrimps found only three extracts to have mildy cytotoxicity with LC50 less than 100 µg/mL

The rest were non toxic with LC50 > 100µg/mL

i. *Tribulus terrestris* chloroform 52.5069 µg/mL

ii. *Rubia cordifolia* aqueous 75.8198 µg/mL

iii. *Conyza bonariensis* methanol 79.0076 µg/mL

Conclusion

Conyza bonariensis and *T. terrestris* have both antimicrobial effects and they are not cytotoxic.

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