PHYTOCHEMICAL COMPOSITION, ANTIBACTERIAL AND ALLELOPATHIC ACTIVITIES OF *Achyranthes aspera* AND *Tagetes minuta* LEAF EXTRACTS

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A THESIS SUBMITTED TO THE SCHOOL OF SCIENCE, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE DEGREE IN MICROBIOLOGY IN THE DEPARTMENT OF BIOLOGICAL SCIENCES, UNIVERSITY OF ELDORET, KENYA

2025

DECLARATION

Declaration by the student

This thesis is my own original work and has never been submitted in any other institutions of higher learning. A list of references is included, and the text appropriately acknowledges the information gleaned from the literature. No part of this thesis may be reprinted without permission of the author and/or University of Eldoret.

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DEDICATION

I dedicate this master research work to my late parents Mr. & Mrs. Philemon Maiyo who were very passionate in education but could not witness this success.

ACKNOWLEGMENTS

My profound gratitude goes to my supervisors Dr. Pascaline Jeruto, Dr. Emily Too and Prof. Lizzy A. Mwamburi of University of Eldoret (UoE) for their efforts, contributions and support towards the completion of this research. To Dr. Nixon Ronoh of Moi University, I appreciate your contribution as well.

My appreciation also goes to my dad Mr. George Philip Lagat, my husband Moses K. Kemboi and our daughter Ariel Jepkoech for their unending prayers, loving guidance, emotional and financial support which made this research and thesis writing not only possible but quite an exciting experience.

I would also like to thank my brothers, friends, classmates and all my relations for their moral support during the entirety of my master's programme.

Finally, thanks and glory be to Almighty God for the good health and care He has given to me throughout my life.

ABSTRACT

Antibiotic resistance remains a significant global health concern to date, putting at risk treatment options. As a result, treatments can be expensive and ineffective. It is therefore necessary to look for new options like using plant extracts with antimicrobial properties. *Achyranthes aspera* and *Tagetes minuta* have been traditionally used to treat various diseases in many communities and may possess antibacterial and allelopathic properties. Weeds cause more crop losses than insects, pests and diseases combined however their antimicrobial and allelopathic properties have not received much attention. This research aimed to determine the phytochemical composition, antibacterial and allelopathic activities of *Achyranthes aspera* and *Tagetes minuta*. Leaves were identified from the farm fields of University of Eldoret, collected, placed in sample collection bags, then transported to the laboratory. The leaves were washed, shade dried then ground to semi-powdery form. Extraction was conducted using sterile distilled water and ethanol. Extracts were tested against *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 51299, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 by disc diffusion method in a Completely Randomized design with three replications. Ciprofloxacin was used as a positive control. Clear zones around the discs were recorded as inhibition zones in millimetres. Maize, millet, rice and sorghum seeds were dressed with extracts to establish their allelopathic activities by placing five surface sterilized seeds in each petri dish. Fifteen millilitres of extracts were used with distilled water set as a positive control. The design of the experiments was Completely Randomized with three replications. ANOVA was used to determine statistical significance at $P \leq 0.05$. *A. aspera* and *T. minuta* extracts showed significant inhibitory effects with inhibition zones of ≥13 mm compared to ≥17 mm from ciprofloxacin. Bio-activity of extracts was highest on *S. aureus* followed by *E. faecalis* then *P. aeruginosa* then *E. coli* with least effect on *K. pneumoniae*. Plumule and radicle lengths of the test plants were also significantly affected with percentage reductions of ≥63%. Extracts were more detrimental on Rice followed by Maize then Sorghum with minimal effect on Millet. Millet recorded the highest germination percentage of 89.44% while rice had the lowest percentage at 67.78%, with Maize (87.50%) and sorghum (85.83%). Alkaloids, coumarins, flavonoids, phenols, quinones, saponins, tannins and terpenoids were present in extracts of both plants while Anthraquinones, glycosides and steroids existed only in *A. aspera*. This study concludes that *A. aspera* and *T. minuta* have remarkable antibacterial and allelopathic activities. There is therefore need to balance between controlling these plants which grow as weeds and maintaining them aimed at utilization for the development of newer antimicrobials and/or bio-control agents.

Keywords: Allelopathy, Antibacterial activity, Medicinal plants, Phytochemicals

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LIST OF ABBREVIATIONS

AMR	–	Antimicrobial Resistance
ANOVA	–	Analysis of Variance
ATCC	–	American Type Culture Collections
CLSI	–	Clinical and Laboratory Standards Institute
CRD	–	Completely Randomized Design
DMSO	–	Dimethylsulphoxide
GVI	–	Germination Vigour Index
MDR	–	Multidrug Resistant
NA	–	Nutrient Agar
PDR	–	Pan-Drug Resistant
SVI	–	Seed Vigour Index
WHO	–	World Health Organization
XDR	–	Extensive Drug Resistant

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Increasing microbial resistance to antibiotics has developed over the past years in both medical and veterinary sectors (Palma *et al.,* 2020). Microbial infections have caused a big burden of diseases. Bacteria are listed in the first position among common microorganisms responsible for infections. Concerns about the issue of microbial resistance and rising antibiotic resistance is spreading throughout the world. Therefore, there is need for the development of microbial drugs which are not only active against these drug resistant microbes, but more importantly kill persistent microorganisms and shorten the length required for treatment (Murugaiyan *et al.,* 2022).

Eighty percent of Africa's populations depend on traditional medicine particularly plant biodiversity for primary health care because plant derived medicine are relatively cheaper and safer compared to their synthetic alternatives (Jamshidi-Kia *et al*., 2018; Anand *et al.,* 2019). This is often attributed to antibiotic resistance as well as preferences by some people. Even when there is access to modern medicine, some still believed in the potency of herbal medicine while some prefer to combine both herbal and conventional medicine (Yuan *et al.,* 2016).

Plants produce a wide range of secondary metabolites such as alkaloids, flavonoids, phenolic compounds, saponins, and tannins that have been reported to offer significant nutritional, biological, and pharmacological benefits to humans (Khalid *et al.,* 2018). These natural compounds can actively inhibit or destroy microorganisms, thereby protecting plants from pathogens including nematodes, bacteria, viruses and fungi (Basit *et al.,* 2021).

Weeds have been shown by various studies to possess medicinal and therapeutic activities and have been utilized in maintaining good health since ancient times (Jamshidi-Kia *et al*., 2018). Plants utilised for medicinal purposes contain many active phytochemicals which have been of great interest in the past current and future studies to obtain newer and more effective antimicrobials both singly or in combinations (Singh, 2015). Many commercial drugs used in modern medicine are plant-derived following ethno-botanical and ethno-medical knowledge and research (Asif *et al.,* 2021).

Most plants with medicinal properties including *Achyranthes aspera* and *Tagetes minuta* which are the focus of this study, are actually weeds. According to Anwar *et al.,* (2019), weeds are a wide set of non-economic plants that grow in undesired areas or alongside cultivated crops, often resulting in economic losses like lower yields due to competition or by releasing allelochemicals, a process known as allelopathy. Allelopathy is a known biological phenomenon in which organism(s) releases one or more allelochemicals that escapes into the environment and influence the germination, survival, reproduction, growth as well as the development of neighbouring plants (Bahadur *et al.,* 2015).

1.2 Statement of the Problem

Antibiotics have played a central role in treating and/or managing infections for quite a long time now. However, widespread antibacterial resistance has been caused by the inappropriate, irregular and irrational uses of antibiotics as well as the inaccessibility and/or unavailability of essential medicine (Anand *et al.,* 2019). The rise in antibiotic resistance has resulted in the decrease of the active antimicrobials available to treat infections resulting from multi-drug resistant (MDR) microbes which are now virtually resistant to nearly all available antibiotic classes (Ventola, 2015). According to Murray *et al.,* (2022),

an estimated five million mortalities were associated with antibacterial resistance in the year 2019 alone. Three million of those fatalities were as a result of antibiotic resistant *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Streptococcus pneumoniae* (Murray *et al.*, 2022).

Due to resistance to the majority of available antibiotics, people are turning to medicinal plants, which humans have long relied upon to largely satisfy medical demands in order to preserve health and cure infectious and/or chronic illnesses (Jamshidi-Kia *et al*., 2018). While some individuals prefer to mix modern and herbal therapy, others continue to trust in the effectiveness of herbal medicine even when modern medication/ pharmaceuticals are accessible (Yuan *et al.,* 2016). About 80% of people in Africa, including Kenyans, get their medications from medicinal plants (Jamshidi-Kia *et al.,* 2018). In addition, they serve as the foundation for a substantial proportion of modern drugs used in the management and/or treatment of various illnesses currently (Hosseinzadeh *et al.,* 2015). Most medicinal plants are known to grow as weeds, *Achyranthes aspera* and *Tagetes minuta* are no exception.

Weeds are plants that either grow alongside crops or in areas where they aren't needed, resulting in economic losses (Anwar *et al.,* 2019). In agricultural systems, Weeds generally affect the yields and quality of the harvest and may lead to increased costs of production, often with large economic impacts. They are particularly detrimental to crop production, causing reduction in terms of quality and quantity through competition and/or allelopathy (Anwar *et al.,* 2019). Studies have estimated the amount of yield losses from weeds to be much higher than pest and disease losses (Zohaib *et al.,* 2016) probably due to the extremely high percentage of allelochemicals they possess. Once released into the environment, their allelochemicals can interact with surrounding organisms affecting the

growth and development of other biological systems by exerting inhibitory and stimulatory effects (Araújo *et al.,* 2021). Additionally, soils infested with weeds tend to exhibit reduced organic matter content, which negatively influences crop yields. Such impacts have significant socioeconomic implications, particularly in Kenya, where approximately 75% of the population depends directly or indirectly on agriculture for their livelihoods (Ochilo *et al.,* 2019). In response, many farmers resort to the excessive use of chemical fertilizers and herbicide primarily to supplement their nutrient requirements and control weeds (Khan *et al.,* 2019). However, they do create hazards for the spray drift and have negative impacts on crops, ground water, soil and environment however, they cannot economically control herbicides resistant weeds (Abbas *et al.,* 2017). Moreover, herbicidal residues in food commodities also directly or indirectly affect human and animal health contributing to life threatening diseases such as cancers (Tanveer *et al.,* 2019).

To date, it is not known whether *Achyranthes aspera* and *Tagetes minuta* have inhibitory effects on germination these grains which are staple foods in Kenyan households. This study therefore investigates the antibacterial activities of *A. aspera* and *T. minuta* and the potential effects of these weedy species on germination of seeds of selected grains.

1.3 Justification

Increased microbial resistance linked with antibiotic resistance as well as unavailability and/or inaccessibility of medicine, or due to undesirable side effects associated with some antibiotics has led to more than 70% of the world's rural population particularly in most developing countries like Kenya, to utilize traditional medicines for primary health care (Jamshidi-Kia *et al*., 2018; Anand *et al.,* 2019). Traditional medicines more often than not are from medicinal plants which are either cultivated or grow naturally as weeds. Weeds

constitute an integral component of many ecosystems. They are a threat to agro-ecosystems despite being reservoir of many vulnerable genes.

According to Kemboi *et al.,* 2022 and Zohaib *et al.,* 2016, weeds causes crop losses even more than those caused by insects, pests and diseases. According to Anwar *et al.,* (2019), weeds interfere with many metabolic processes resulting from weed-crop competition and allelopathy, which alters normal growth and lowers agricultural output. Zohaib *et al.,* 2016 estimated weeds to be capable of causing a decline of up to 35-80% in maize yields, 35-40% reduction in rice yield and 25-30% in the yield of wheat.

Some studies have reported yield losses which results from weed-crop allelopathic interactions (Kemboi *et al.,* 2022) as well as the effectiveness of medicinal plants in managing and/or treating various bacterial infections and diseases (Yuan *et al.,* 2016). Their effectiveness may be attributed to their secondary metabolites/ phytochemicals. However, there exists limited information that highlights the detrimental impact of weeds on seed germination owing to weed-crop allelopathic interactions although losses from weeds have been estimated to be even greater than those from insect pests and diseases (Kemboi *et al.,* 2022). This is to a higher percentage attributed to the production and release of allelochemicals from leaves, flowers, seeds, stems and roots of plants, an alternative to chemical herbicides (Arora *et al.,* 2015). These environmentally safe substances, once they are released to the environment, they interact influencing the growth and development of biological systems, including inhibition and/or stimulation effects (Tanveer *et al.,* 2019). However, their inhibitions effects are often hazardous to many ecosystems.

Since a single plant has a vast range of phytochemicals, resistance to such botanicals is believed not to be as widespread as in antibiotics and chemical herbicides which have a few active ingredients (Ruddaraju *et al.,* 2020). *Achyranthes aspera* and *Tagetes minuta* may exists/grow as weeds but have been documented to potentially possess antibacterial and allelopathic activities by earlier studies (Arora *et al.,* 2015; Habtamu & Mekonnen, 2017; Santos *et al.,* 2017; Tanveer *et al.,* 2019). There is therefore need to balance between controlling these plants (medicinal plants) as weeds and maintenance aimed at for utilization purposes.

To the best of my knowledge, the information highlighting the detrimental effects of weeds on crops as a result of weed-crop allelopathic interactions as well as effectiveness of some medicinal plants is not exhaustive. Weeds like *Achyranthes aspera* and *Tagetes minuta* in this case, have not been studied conclusively. The current study therefore sets to explore and document the antibacterial activities of *Achyranthes aspera* and *Tagetes minuta* against selected pathogens as well as their allelopathic effects on the germination of some seeds of some food crops.

1.4 Objectives

1.4.1 Broad objective

To determine the antibacterial activities, allelopathic effects and phytochemical composition of *Achyranthes aspera* and *Tagetes minuta* leaf extracts.

1.4.2 Specific objectives

i. To determine antibacterial activities of *Achyranthes aspera* and *Tagetes minuta* aqueous and ethanolic leaf extracts against *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 51299, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213.

ii. To determine the allelopathic effects of *Achyranthes aspera* and *Tagetes minuta* aqueous and ethanolic leaf extracts on germination of maize, millet, rice and sorghum seeds.

iii. To qualitatively determine the phytochemical compound(s) present in *Achyranthes aspera and Tagetes minuta* aqueous and ethanolic leaf extracts.

1.5 Hypothesis

1. Crude leaf extracts of *Achyranthes aspera* and *Tagetes minuta* are not active against *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 51299, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213.
2. Crude leaf extracts of *Achyranthes aspera* and *Tagetes minuta* do not inhibit germination of maize, millet, rice and sorghum seeds.
3. Crude leaf extracts of *Achyranthes aspera* and *Tagetes minuta* do not possess active phytochemicals.

CHAPTER TWO

LITERATURE REVIEW

2.1 Medicinal Plants of Interest

The majority of the more than 200,000 known natural plant-based compounds are derived from higher plants as well as microorganisms (Habtamu & Mekonnen, 2017; Sorokina & Steinbeck, 2020). The need for pharmaceuticals to treat the increasing number of ailments and our expanding population is driving researchers to constantly look for new and more potent drug sources. One of the top priorities in the search for natural products for the pharmaceutical sector is medicinal plants (Boy *et al.,* 2018). *Achyranthes aspera* and *Tagetes minuta* grow as weeds in the fields but have been documented to have medicinal properties as well as herbicidal potential (Arora *et al.,* 2015; Habtamu & Mekonnen, 2017; Santos *et al.,* 2017; Jan *et al.,* 2019; Tanveer *et al.,* 2019; Safdar *et al.,* 2021; Sharma *et al.,* 2022; Sidhu *et al.,* 2023).

Any plant utilized in contemporary or traditional medicine on a local or regional level that can be utilized for a particular illness, used to preserve health, or both is considered a medicinal plant (Ahn, 2017). The use of traditional medicine has skyrocketed over the last 20 years, with the WHO even encouraging the use of medicinal plants due to their shown efficacy, safety, lack of toxicity, accessibility, and dependability (Sen & Chakraborty, 2017). Over 80% of the world's population’s main source of primary healthcare is mostly obtained from medicinal plants, according to the World Health Organization (Jamshidi-Kia *et al*., 2018).

2.1.1 *Achyranthes aspera*

Achyranthes aspera Linn. (devil's horsewhip) (Plate 2.1) is a weed of the family *Amaranthaceae* and is widely distributed in the tropical and subtropical regions. It can potentially grow on undisturbed waste land and a major weed of cultivated crops including maize, sugarcane and wheat (Tanveer *et al.*, 2019).

Plate 2.1: *Achyranthes aspera* L. (Source: Author, 2024).

This perennial stiff erect herb, 2.0 m high grows up to 100 cm in height. Stems are square, leaves elliptic ovate or broadly rhombate, 5-22 cm long, 2.5 cm broad, and adpressed pubescent. The inflorescences are 8 - 30 cm long, with many single, white or red flowers, 3-7 mm wide. Flowering time is in summer. The main root is long cylindrical thick; secondary and tertiary roots present slightly ribbed, yellowish-brown in colour; odour is slight, the taste is slightly sweet and mucilaginous; the stem is yellow-brownish, erect branched, cylindrical hairy about 60 cm high. Seeds are subcylindrical, truncates at apex,

rounded at base, black, and shining (Sharma & Chaudhary, 2015). *Achyranthes aspera* plants contain various phytotoxic compounds that can potentially work as herbicides to inhibit germination and growth of other weed species (Abbas *et al.*, 2017).

2.1.2 *Tagetes minuta*

Tagetes minuta (wild marigold) (Plate 2.2) is a weed in the family *Asteraceae* and grows up to a height of 1-2 meters. It is made up of blued-green stems up to 15 cm long, with 19–17 leaflets and small creamy yellow flowers which are about 10 mm long and 2 mm wide. This herbaceous plant is commonly found in Africa but some species have become naturalized around the world (Verma *et al.*, 2024). *Tagetes minuta* is a rather hardy plant having a 120–150-day lifespan with a respectably long shelf life. It grows in the summer, winter, and rainy seasons. Although seeds are the main form of propagation, vegetative cuttings have also been used recently. This species' blooms are typically found in vivid shades that vary from yellow to orange (Sharma *et al.*, 2022).

Plate 2.2: *Tagetes minuta* L. (Source: Author, 2024).

Through a variety of techniques, the phytochemicals found in this plant have been shown to be effective deterrents of a number of bacteria, trematode nematodes, roundworms, human fungus, and fungi (Yeasmin & Gupta, 2022). Some components contained in *Tagetes minuta* extracts such as flavonoids have been shown to have antibacterial activity against not only bacteria but also fungi and some nematodes (Santos *et al.,* 2017).

2.2 Uses of Achyranthes aspera and Tagetes minuta

2.2.1 Uses of *Achyranthes aspera*

Achyranthes aspera has long been utilised traditionally to treat a variety of diseases, including odontologic, rheumatism, bronchitis, skin conditions, rabies, fever, diarrhea, diabetes, and antifungal and antibacterial properties (Habtamu & Mekonnen, 2017). According to research conducted in India, *A. aspera* has a number of significant therapeutic qualities, including antiperiodic, diuretic, purgative, laxative, antiasthmatic, hepatoprotective, and anti-allergic effects (Habtamu & Mekonnen, 2017). According to Mishra (2018), it is particularly used as a spermicidal, antipyretic and as a cardiovascular agent. In Ethiopia, fresh *A. aspera* L. leaves soaked in water are traditionally utilized to cure skin infections. Additionally, Ethiopian locals also use the leaves to treat wounds and to halt the bleeding (Habtamu & Mekonnen, 2017).

Regular consumption of a handful of *A. aspera* seeds helps manage weight by reducing excess fat accumulation, which results in a reduction of bodyweight (Christi *et al.,* 2022). Essential oils extracted from the leaves of *Achyranthes aspera* possesses strong prophylactic potentials, anti-cancer, anti-microbial, anti-diabetic, diuretic, hepatoprotective, antioxidant, anti-inflammatory, anti-arthritic, cardio-protective,

immuno-modulatory and prothyrodic activity thus used in several medicinal formulations to treat severe illnesses (Rehman *et al.*, 2018).

Achyranthes aspera L. is highly esteemed by traditional healers and used in treatment of asthma, bleeding, in facilitating delivery, boils, cold, cough, colic, debility, dropsy, dog bite, dysentery, ear complications, headache, leucoderma, pneumonia, renal complications, scorpion bite, snake bite, and skin diseases etc. Traditional healers claim that addition of *Achyranthes aspera* would enhance the efficacy of any drug of plant origin (Ganesh *et al.*, 2021).

Mary & Giri, (2017) carried out GC-MS analysis of ethanolic leaf extracts of *A. aspera* and reported that some of the identified compounds possess various biological activities such as antimicrobial, antioxidant, antiseptic, pesticide, fungicide, diuretic, anti-inflammatory and anticancer.

2.2.2 Uses of *Tagetes minuta*

Several traditional uses of the marigold plant's various components have been documented, in addition to its commercial usage as an ornamental plant. Extract from leaves has antioxidant and anti-inflammatory properties (Sharma *et al.,* 2022). The strong-smelling essential oils from *Tagetes minuta* have enabled it to be used for many purposes, including as a relish, laxative, diuretic, flavouring, insect repellent, stimulant and snuff (Akram & Tembhre, 2016). *T. minuta* is grown as a vegetable in parts of Peru, dried leaves are utilised as condiments and flavouring in different food products. It is also utilised for the treatment of coughs, stomach cramps and rheumatism (Akram & Tembhre, 2016). The leaves are used against muscular-pain, piles, ulcers, wounds and kidney troubles (Kar & Patra, 2022).

Tagetes minuta plant is used to flavour candies, drinks, milk, cheese, baked goods, sweets, and gelatins. It is also used in cooking (Yeasmin & Gupta, 2022). The entire plant is used as a diaphoretic, purgative, hysterical cure, condiment, and stomach strengthener. The plant's leaves are used to treat inflammatory, microbiological, bronchodilatory, and wound-healing disorders because it has many components that serve distinct purposes. It can also be utilised in treating muscle soreness and renal issues. On the other hand, leaf extracts can be used as snuff, pesticide, earache and haemorrhoids. Its flowers are utilised in treating epileptic fits, fevers, indigestion, gastritis, and mild laxatives (Yeasmin & Gupta, 2022).

T. minuta has been utilised in treating colic, diarrhoea, vomit, fever, skin diseases, hepatic disorders and also useful in eye health protection (Sharma *et al.*, 2022). *T. minuta* extracts are ingredients of medicinal drugs used to treat common cold, inflammation, bowel and stomach illnesses, skin infections, cough, cold and wounds (Latıfian *et al.*, 2021).

Since pre-contact times, *Tagetes minuta* has been consumed in a variety of ways. Huacatay paste is utilised in preparing the well-known Peruvian potato dish ocopa, and dried leaves can also be used as a flavouring agent. Leaves can be used to make a herbal tea. "Marigold oil" is extracted and utilized in the soft drink, tobacco and perfume industries. Apart from being used as food, the plant may also be used in making dyes, as a green manure crop for biomass, and as a bio-fumigant to suppress some worm species (Yeasmin & Gupta, 2022).

Traditionally, it has been used in treating a number of ailments, including respiratory issues, stomach disorders, colds, as they are sedatives, insecticides, antispasmodics, anti-septic and anti-parasitic (Shirazi *et al.*, 2014). *T. minuta* significantly reduced tomato early blight caused by *Alternaria solani* when intercropped with tomatoes (Sharma *et al.*, 2022).

2.3 Antibacterial activity

2.3.1 *Achyranthes aspera*

Ahmad *et al.,* (2022), tested eight different antibiotics against *Acinetobacter baumannii*, Methicillin-resistant *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Enterococcus faecalis* and *Pseudomonas aeruginosa*. For Methicillin-resistant *Staphylococcus aureus* Cefoxitin, Penicillin, and Co-trimoxazole were resistant out of seven antibiotics. The authors observed some degree of resistance to Ciprofloxacin, Levofloxacin, Penicillin, Amoxicillin, Imipenem, Ceftriaxone, Ceftazidime and Vancomycin. However, after a combination of these antibiotics with leaf extracts of *Achyranthes aspera*, the zones of inhibition for all these antibiotics goes from the resistant and/or intermediate to the sensitive range. This suggests that the *Achyranthes aspera* leaf extracts can reverse antibiotic resistance naturally without any side effects on the human body.

Trypsin inhibitor (AATI) isolated from the seeds of *Achyranthes aspera* significantly affected the growth of *Proteus vulgaris* followed by *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumonia* with zones of inhibition recorded as 28 mm, 26 mm, 25 mm, 20 mm and 14 mm, respectively (Ganesh *et al.,* 2021).

Petroleum ether and methanolic leaf extracts of *A. aspera* at different concentrations were shown to be active against nine different bacteria (*M. luteus*, *B. subtilis*, *S. mitis*, *E. coli*, *P. aeruginosa*, *K. pneumonia*, *S. typhi*, *S. paratyphi* A (MTCC-3220), *S. flexneri*) (Mishra *et al.,* 2020). The ethanol extracts from *Achyranthes aspera* was found to be effective against *Salmonella typhimurium, Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa* and *Salmonella abony* strains (Mishra, 2018). The acetone extracts of *A. aspera* demonstrated remarkable antibacterial activity against *Escherichia coli, Salmonella typhimurium,*

Staphylococcus aureus, *Bacillus subtilis* and *Pseudomonas aeruginosa* strains (Mishra, 2018). Similarly, ethyl acetate extracts of the same plant exhibited notable inhibitory effects against *Salmonella typhimurium*, *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* strains (Mishra, 2018). In another study, methanolic extracts prepared from the whole dried plants of *A. aspera* were tested against *Escherichia coli*, *Klebsiella pneumoniae*, and *Bacillus cereus*. Among these, *E. coli* showed the highest zone of inhibition (24 ± 0.5 mm), followed by *K. pneumoniae* (23 ± 0.7 mm) and *B. cereus* (20 ± 0.51 mm) at a concentration of 2000µg, indicating strong antibacterial potential. All the bacterial species tested were found to be susceptible to the methanolic extracts (Ganesh *et al.,* 2021). Additionally, *A. aspera* exhibited an inhibition zone of up to 14 mm against *Pseudomonas* species using the well diffusion method (Habtamu & Mekonnen, 2017).

2.3.2 *Tagetes minuta*

According to Shahzadi & Shah, (2015), Butanol and ethyl acetate extracts of flowers and seeds from *T. minuta* exhibit significant antibacterial activity against *Micrococcus leteus*, *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas pikettii*. Essential oils from *Tagetes minuta* L. have been documented to completely inhibits the growth of *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Streptococcus agalactiae* ATCC 27956, *Staphylococcus epidermidis*, *Acinetobacter baumannii* ATCC 19606, *Escherichia coli* ATCC 25922 and *Enterobacter cloacae*. However, *Salmonella enterica* sv. *Typhimurium* ATCC 13311, *Shigella sonnei* ATCC 9290, *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC 700603 all showed growth all be it suppressed (Abdoul-Latif *et al.,* 2022).

Opinde *et al.* (2018) examined leaf extracts from *T. minuta* reported that they showed antimicrobial activity against clinical isolates of *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, *Shigella flexineri*, *Enterococcus faecalis* with inhibition zones ≥17.00mm against all test microorganisms.

Ali *et al.* (2014) investigated *T. minuta* leaf extracts' essential oil against *Pseudomonas aeruginosa* ATCC 25619, *Staphylococcus aureus* ATCC 29737, *Escherichia coli* ATCC 10536, *Bacillus subtilis* ATCC 6633, methicillin-resistant *S. aureus* (MRSA) and documented that they exerted remarkable antibacterial activity with the inhibition zones of up to 23mm.

Jan *et al.* (2019) investigated the essential oil of the flowering shoot of *Tagetes minuta* L., against *B. subtilis* ATCC 6633, *E. coli* ATCC 25922, clinical isolate of *Shigella flexenari*, *Salmonella typhi* ATCC 19430, *S. aureus* ATCC 25923 as well as *Pseudomonas aeruginosa* ATCC 27853. The authors reported antibacterial effect of *T. minuta* extracts with zone of inhibition ranging from 22mm and 20mm.

Bacterial strains like *Escherichia coli*, *Staphylococcus aureus*, *Micrococcus leteus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Pseudomonas savastanoi*, *Pseudomonas pikettii* and *Xanthomonas axonopodis* have also been reported to be inhibited by extracts of *T. minuta* (Walia, & Kumar, 2020).

Panwar & Bhatt, (2014) also reported that *Tagetes minuta* L. has antibacterial activities against *Staphylococcus aureus* as well as *Streptococcus pyrogenes*. Tahir & Khan, (2012) have documented the antibacterial potential of crude leaf, fruit and flower extracts of *Tagetes minuta*.

2.4 Bacteria of Interest

2.4.1 *Escherichia coli*

Escherichia coli is gram negative bacteria in the *Enterobacteriaceae* family. *E. coli* is a non-sporulating facultative anaerobe. Virulent strains differ from nonvirulent *E. coli* by possessing genetic elements for virulence factors (Ou *et al.,* 2024). Different somatic (O) and flagellar (H) antigens and unique virulence traits are present in each class of *E. coli*. These strains include Shiga toxin-producing *E. coli* (STEC), notably *E. coli* O157:H7, enteroinvasive *E. coli* (EIEC) and enterotoxigenic *E. coli* (ETEC) (Makvana & Krilov, 2015). Because *E. coli* frequently carries drug-resistant plasmids and swiftly spreads them to other species under stress, it serves as a significant reservoir for transmissible antibiotic resistance (Makvana & Krilov, 2015). It contributed to the more than three million mortalities associated with antibacterial resistance worldwide in the year 2019 (Murray *et al.,* 2022). Numerous diverse diarrheal diseases are linked to various *E. coli* strains. Transmission is by the faecal-oral route. Pili (fimbriae) allow the bacteria to colonize the ileal mucosa. Infection is common where sanitation is poor; both infants and susceptible travellers to developing countries are particularly at risk. The disease is most serious in infants (Adler *et al.,* 2022). Diagnosis is by stool culture with prevention involving sanitary measures like hand-washing and proper preparation of food, chlorination of water supplies, sewage disposal and treatment.

2.4.2 *Enterococcus faecalis*

Enterococcus faecalis is a Gram negative, commensal enteric bacterial pathogen usually located in the intestinal tracts of either animals or human beings. Due to its ability to thrive in harsh environmental and nutritional circumstances, this facultative anaerobic coccus can

be detected in resistant endodontic infections (de Siqueira *et al.,* 2022). It is part of the normal flora in the human gastrointestinal tract. The pathogenic members of enteric bacteria are usually associated with infections that are characterized by enteric fevers, abdominal pain and diarrhoea and vomiting (Rachuonyo *et al.*, 2016). The primary mode of transmission is through the fecal-oral route. In healthcare settings, transmission can also occur through contaminated medical equipment (Caliman-Sturdza, 2024). Cytolysin, a pore-forming exotoxin that lyses both bacterial and eukaryotic cells, is produced by highly virulent strains of *Enterococcus faecalis* (Parga *et al.,* 2023). Diagnosis is often by blood culture. Treatment of enterococcal infections often involves antibiotics, and the choice of antibiotics is guided by susceptibility testing. Due to increasing antibiotic resistance, combination therapy or the use of specific antibiotics may be necessary (Caliman-Sturdza, 2024).

2.4.3 *Klebsiella pneumoniae*

Klebsiella pneumoniae is an encapsulated, non-motile, facultatively anaerobic, gram-negative bacteria (Chang *et al.,* 2021). They can be found in water, soil, and any other surfaces in nature. *K. pneumoniae* commonly colonizes the digestive and nasal tracts of individuals without producing any symptoms. However, if the host immune system is unable to stop the pathogen's development, the colonization might lead to a serious infection if it spreads to other areas of the body such as the lungs even though it is mostly benign in the intestines (Martin & Bachman, 2018). Pneumonia, urinary tract infections, meningitis, and bloodstream infections are examples of infections which can result from that uncontrolled spread. *K. pneumoniae* is attributed to the more than three million mortalities associated with antibacterial resistance in the year 2019 (Murray *et al.*, 2022).

The primary method in most hospitals to screen for presence of *K. pneumoniae* still remains specimen culture (Chang *et al.,* 2021). The main prevention and control measures in limiting the uncontrolled spread of *K. pneumoniae* include using single-use devices to reduce transmission from contaminated equipment, practicing good hand hygiene, removing medical devices like catheters and tubes when they are no longer needed. Isolating patients who are colonized or infected with highly antibiotic-resistant *Klebsiella* strains and actively monitoring patients at risk for *Klebsiella* carriage and infection is also key (Li *et al.,* 2019).

2.4.4 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a member of the *Pseudomonadaceae* family and is a non-fermenting gram-negative rod. One of the main organisms responsible for nosocomial infections, *P. aeruginosa* mainly affects patients with compromised immune systems or those hospitalized to the intensive care unit. It can occupy a wide range of ecological niches, particularly in damp habitats. Meningitis, pneumonia, and urinary tract infections are only a few of the potentially fatal illnesses that people can contract from the well-known opportunistic bacteria *P. aeruginosa* (Sandhya, 2016). It may acquire resistant genes horizontally or through chromosomal alterations to become resistant. Additionally, they can be intrinsically resistant to many antimicrobials causing bacteraemia, severe pneumonia or even deaths (Ruiz-Garbajosa & Canton, 2017; Mohammed & Abdullah, 2020). It has been documented that *P. aeruginosa* contributed to the more than three million mortalities associated with antibacterial resistance in the year 2019 (Murray *et al.,* 2022).

2.4.5 *Staphylococcus aureus*

Staphylococcus aureus is an aerobic and facultative anaerobic gram-positive bacilli of the family *Staphylococcaceae*. This catalase and coagulase positive commensal colonize 30% of healthy individuals from different body parts like the anterior nares' skin and mucous membrane, the perineum, genitourinary tracts, and even the pharynx (Mohammed & Abdullah, 2020). *S. aureus* is a major cause of nosocomial and community-acquired infections. It also contributes to a variety of illnesses in both humans and animals that have a huge on public health concern (He & Wunderink, 2020). It plays a very significant role in causing infections in humans and animals both in hospitals and the community ranging from simple to life threating infections. *S. aureus* causes superficial skin lesions (boils, styes), localized abscesses in other sites as well as deep-seated infections (Howden *et al.,* 2023). They are a significant contributor to hospital-acquired infections that are increasingly resistant to all kinds of available antibiotics (Rasheed & Hussein, 2021). It contributed to the more than three million mortalities associated with antibacterial resistance in the year 2019 (Murray *et al.,* 2022). Definitive diagnosis of *S. aureus* infection is made by obtaining a culture from the area of suspected infection (Azad & Patel, 2024). Some skin infections do not require treatment. Skin infections caused by *S. aureus* may require incision and drainage of the infected site and/or antibiotic treatment. Hand washing, cleaning and disinfecting frequently touched surfaces, using barriers like a towel or clothing between your skin and shared surfaces as well as regular washing all clothing are some commonly used control and preventive measures.

2.5 Antimicrobial resistance (AMR)

Antimicrobial resistance (AMR) is the process by which microbes develop resistance to previously effective antimicrobials over time commonly resulting from inappropriate and irregular use of antimicrobial drugs as well as pathogen mutations (Ventola, 2015). The unavailability of efficient and effective antimicrobial drugs has rendered many common infections increasingly challenging to treat with individuals remaining sick longer hence extended hospital stays and an increase in morbidity and mortality (O'Neill *et al.,* 2016).

AMR arises from three major factors that includes the introduction of harmful pathogens into the environment by humans, increase in the prevalence of resistant phenotypes among microorganisms and the widespread, typically needless use of antibiotics, which exerts selective pressure that drives evolution of microorganisms (Michael *et al.,* 2014). Target antimicrobial resistance may develop in almost any microbe at any moment. Resistance to antibiotics is acquired by bacteria, resistance to antifungals by fungi, resistance to antivirals by viruses and resistance to antiprotozoal drugs by protozoa (Anand *et al.,* 2019).

Antimicrobial resistance can manifest as pan-drug resistance, extensive drug resistance, or multi-drug resistance. Multi-drug resistance (MDR) describes the acquired inability to react to at least one antimicrobial agent from three or more antimicrobial classes. Unlike extensive drug resistance (XDR), which is the acquired non-susceptibility to at least one antimicrobial agent in all but one or two antimicrobial classes, pan-drug resistance (PDR) is the acquired non-susceptibility to all antimicrobial agents in all classes (Sweeney *et al.,* 2018). *Achyranthes aspera* leaf extracts can reverse antibiotic resistance naturally without any side effects on the human body (Ahmad *et al.,* 2022).

2.6 Allelopathy

Allelopathy can be defined as a biological process via which an organism generates one or more allelochemicals that escapes into the environment to influence the germination, survival, reproduction, as well as growth and development of neighbouring organisms (Kemboi *et al.*, 2022). The allelopathic compounds are chemicals produced by some plants (especially medicinal and aromatic plants) that can affect the ecosystem in association with other compounds in collaboration with microorganisms (Sadeqifard *et al.*, 2022).

Secondary metabolites known as allelochemicals are released from some plants and have a direct or indirect effect on processes like the germination of other sensitive plants (Nazir *et al.*, 2014). Allelochemicals have negative consequences and are often divided into two categories: functional allelopathy and genuine allelopathy. When compounds that are naturally harmful are released from their source as they are created in plants, this is known as genuine allelopathy. The release of harmful chemicals as a result of microbial transformation is known as functional allelopathy (Kaliyadasa & Jayasinghe, 2018). Plants having allelopathic traits can compete with weeds that grow alongside agricultural crops hindering crop development and production (Casimiro *et al.*, 2017).

Weeds are a wide variety of plants that either grow alongside crops or in areas where they are not needed, resulting in economic losses (Anwar *et al.*, 2019). They constitute integral components of many ecosystems. Besides being reservoirs of many vulnerable genes, they are a threat to agro-ecosystems causing high expenses for agricultural systems (Sadeqifard *et al.*, 2022). They are a threat to crop production causing qualitative and quantitative yield reductions as a result of competition and/or allelopathy (Anwar *et al.*, 2019).

Field crops like maize, millet, rice and sorghum are an important part of diet in almost every meal worldwide. Allelochemicals liberated from the weeds are likely to influence germination of seeds, seedling growth, development and establishment and is regarded as one of the crucial component(s) of an invasive species' success in natural and agricultural habitats (Kimura *et al.,* 2015). *Achyranthes aspera* and *Tagetes minuta* which grow as weeds in the fields, have been documented to possess allelopathic potential (Arora *et al.,* 2017; Tanveer *et al.,* 2019). Their impact increases with increase in the extract(s) concentrations (Kemboi *et al.,* 2022).

2.6.1 Allelopathic activities of *Achyranthes aspera*

The effect of *A. aspera* leaf extracts were discovered to be influenced by the growing matrix in addition to the extract concentrations. In both laboratory and greenhouse settings, the response from rice seeds and seedlings to various extracts varied. It was discovered that *A. aspera* leaf extracts inhibited root length, dried biomass, germination percentage, Germination Vigour Index (GVI), but not Seed Vigour Index (SVI) or shoot length on petri plates. However, in the greenhouse research, it had no influence on any of these parameters (Sidhu *et al.,* 2023).

Tanveer *et al.* (2014) conducted germination bioassays in a lab setting to examine *Achyranthes aspera* L.'s allelopathic suppression of pearl millet, sorghum and maize. When compared to a control treated with distilled water, extracts of every plant parts significantly decreased the germination index, germination percentage, mean germination time as well as seedling root length. The root extract of *A. aspera* showed the highest germination inhibition, causing 0% germination in pearl millet and maize, and the lowest germination rate (6.25%) in sorghum. The crops' mean germination time, germination index and

seedling root length responded differently to extracts from the whole plant, fruit, leaf as well as stem.

In a laboratory setting, Sharma & Satsangi, (2012), examined the allelopathic effects of *Achyranthes aspera* (L.) on germination and growth behaviour of rice (*Oryza sativa* (L.). Weed powder toxicity was shown to have an adverse influence on rice germination and growth. After a 15-day incubation period, the results showed that varying concentrations of *Achyranthes aspera* L. leaf extracts (25%, 50%, 75%, and 100%) significantly inhibited the germination as well as root and shoot elongation of paddy (*Oryza sativa* L.) seeds, while distilled water used as control showed the highest germination. Additionally, the inhibitory impact of these extracts was found to be proportionate to the extract concentrations. The higher the extract concentration, the lower the germination percentage.

The impacts of the leaf biomass of *Achyranthes aspera* L., *Cassia obtusifolia* L. and *Parthenium hysterophorus* L. on wheat and pea seed germination and seedling growth were examined by Gupta & Narayan, (2010). The growth of pea and wheat was greatly impacted by the biomass of weed leaves. They had crop-specific effects, though, and were reliant on leaf biomass quality and dosage. Higher levels of *A. aspera* leaf biomass was shown to have an inhibitory effect on the germination of pea and wheat seeds. The author also explains the possibility of variable effects (additive, antagonistic and synergistic) on the crop growth resulting from the mixtures of the weeds.

Safdar *et al.,* (2021) laid out an experiment to check the allelopathic potential of plant residues mixed with soil and aqueous extract of *A. aspera* flower against six broad leaf weeds of *Rhynchosia capitata, Convolvulus arvensis, Parthenium hysterophorus,*

Trianthema portulacastrum, *Digera arvensis* and *Chenopodium album*. Aqueous extracts and decomposed prickly chaff flower plant material with concentrations of 2, 4 and 6% (w/w) were mixed into the soils that were used as germination media for weeds. Results showed that treatments considerably reduced the seedling establishment of target weeds. Substantial inhibition of target weeds was noted in germination percentage (76.7%), emergence index (75.9%), emergence energy (38.6%) and mean emergence time of 42 days were recorded.

2.6.2 Allelopathic activities of *Tagetes minuta*

The allelopathic effects of *T. minuta*'s volatile oil on *Phalaris minor* Retz., *Amaranthus viridis* L. and *Chenopodium murale* L., three additional invasive weeds, were examined by Arora *et al.* in 2015. *T. minuta*'s volatile oil was found to dramatically and dose-dependently lower the recipient weeds' germination, growth, chlorophyll content, and respiratory capacity. In cells of treated root tips, mitotic investigations showed a total cessation of mitotic activity.

Arora *et al.,* (2017) examined the effect of various concentrations of *T. minuta* oil (0.25, 0.5, 1, 2, 3, 4, and 5 µl/ml) on weeds (*E. crus-galli, B. pilosa, C. murale, P. minor, A. viridis* and *A. tricolor*) by laboratory bioassay. Germination percentage, radical length and plumule length of seedlings in all the test weeds was inhibited by *T. minuta* oil. The effect was maximum in *Amaranthus tricolor* with complete inhibition at 1 µl/ml, while *Echinochloa crus-galli* was least affected with complete inhibition at 5 µl/ml concentration of *T. minuta* oil.

The study by Shakkira, (2022) evaluated allelopathic effect of *Andrographis paniculata, Plectranthus ambonicus* and *Tagetes minuta* extracts on rice, cowpea and green gram. Cold water, hot water and methanol extracts of these plants were used in petri plates to test the phytotoxic activity on test crops (cowpea, green gram and rice). Germination indices and seedling growth parameters of test crops were adversely affected by the application of allelopathic extracts. Cowpea and green gram were more sensitive to allelopathic extracts than rice. A notable delay in germination of tested crops, in shoot and root length, and in fresh and dry weights were observed by the application of allelopathic treatments.

Sadia *et al.,* (2015) tested the allelopathic effect of aqueous leaf extracts of *T. minuta* at concentrations of 50%, 75% and 100% on root length, shoot length, germination, fresh and dry weight of Johnson grass and Sun spurge. The results showed reduction in germination, root and shoot growth, fresh and dry weight at all concentrations of *T. minuta* leaf extracts.

2.7 Phytochemical composition of *Achyranthes aspera* and *Tagetes minuta*

Plants protect themselves from the continuous attack of naturally occurring insect pests, pathogens and environmental stresses by producing several compounds termed phytochemicals, as a result secondary metabolism (Boy *et al.,* 2018). Phytochemicals are secondary metabolites which occur naturally and are synthesized by plants during their normal growth and development. They play a crucial part in their defense system, including disease prevention, and are synthesised by medicinal plants, vegetables and fruits during primary and secondary metabolism. Numerous phytochemicals have proven dietary, biological, and medicinal properties (Khalid *et al.,* 2018). The most common phytochemicals in plants are alkaloids, flavonoids, tannins, coumarins, terpenoids,

saponins, steroids, phenols, quinones, cyanogenic clycoside and glucosinolates (Boy *et al.*, 2018; Chanda *et al.,* 2019; Sivakumar, 2022).

2.7.1 *Achyranthes aspera*

The qualitative screening for secondary metabolites (phytochemicals) in *Achyranthes aspera* by Nigussie *et al.,* (2021) indicated the presence of alkaloids, phenols, flavonoids, terpenoids, tannins and steroids in methanolic leaf extracts. The major chemical constituents are carbohydrates, protein, glycosides, alkaloids, tannins, saponins, flavonoids and lignin (Ganesh *et al.,* 2021).

Tiwari *et al.,* (2018) conducted qualitative study of *Achyranthes aspera* and documented that there exist several secondary metabolites. Tannins were found to be present in shoot and root of all extracts prepared by using solvents (methanol, ethanol, acetone, water and diethyl ether). Alkaloid were present in methanolic, ethanolic and water extracts. Phenol was absent in all extracts. Leaf and inflorescence contained flavonoids. Saponins were present in root and stem parts while Coumarins was found in all extract except diethyl ether. Coumaric acid has been detected in extracts of *Achyranthes aspera* through high-performance liquid chromatography (HPLC) – analyses (Tanveer *et al.,* 2014).

According to Srivastav *et al.,* (2011) extracts of *Achyranthes aspera* contain Saponins A (D-glucuronic acid) and B (β-D galactopyranosyl ester of D-Glucuronic acid) along with oleanolic acid, amino acids and hentriacontane. Phytochemical screening of *A. aspera* root extracts revealed presence of alkaloids, tannins, cardiac glycosides, steroids, flavonoids, terpenoids, reducing sugar and saponin (Sharma & Chaudhary, 2015).

Two new bis-desmosidic triterpenoid saponins were isolated, besides the three known saponins from the methanolic extract of the aerial parts of *Achyranthes aspera*. Their structures were elucidated by GC-MS as β-D-glucopyranosyl3β-[O-α-L-rhamnopyranosyl-(1→3)-O-β-D-glucopyranuronosyloxy] machaerinate, β-D-glucopyranosyl3β-[O-β-D-galactopyranosyl-(1→2)-O-α-Dglucopyranuronosyloxy]machaerinate. The other saponins were identified as β-D-glucopyranosyl-3β[O-α-L-rhamnopyranosyl-[1→3)- O-β-D-glucopyranuronosyloxy] oleanolate, β-D-glucopyranosyl3-β-[O-β-D-galactopyranosyl (1→2)-O-β-D-glucopyranuronosyloxy] oleanolate, β-D- glucopyranosyl 3β-[O-β-Dglucopyranuronosyloxy] oleanolate (Ghimire *et al.*, 2015)

Numerous phytochemicals are found in *A. aspera* leaves. However, their contents vary from one place to another due to the soils and climatic conditions. Leaf extract's GC-MS analysis of *A. aspera* leaf extracts revealed the presence of flavonoid (3,5- Dihydroxy-6-Methyl-2,3- Dihydro-4H-Pyran-4-one), a terpene alcohol [(2E)-3,7,11,15-Tetramethyl-2-hexadecen-1-ol], a natural sygar [2-Furaldehyde,5- (Hydroxymethyl)], a diterpene named Phytol, triterpene called Squalene and Stigmasterol (phytosterol) (Nargatti *et al.*, 2021). The authors also found the seeds of *A. aspera* to contain triterpenoid Saponins A (D-glucuronic acid) and B (β-D galactopyranosyl ester of D-Glucuronic acid).

Hayyat *et al.* (2020) showed that *A. aspera* is reservoir of cardiac glycosides, polyphenols, diterpinoid, steroids, alkaloids, triterpenoid, seuiterpnen lactones and sapogenins. Anand *et al.* (2017) identified three terpenoid compounds namely, ursolic acid, corrosolic acid and achyrantheric acid from petroleum ether extracts of *A. aspera* roots.

According to Abhang, (2024) phytochemical screening of extracts of *A. aspera* obtained from leaves revealed the presence of alkaloids, flavonoids, phenolic compounds, tannins, saponins and terpenoids.

2.7.2 *Tagetes minuta*

Essential oils of *Tagetes minuta* have yielded a wide range of chemical components. These include phenolic compounds, carotenoids, flavonoids, terpenoids, and thiophenes. Compounds such as syringic acid, thienyl terpenes, phenolic compounds, lutein, quercetin, quercetagetin and a glucoside of quercetagetin are the significant phytochemical elements found in the various plant parts (Kar & Patra, 2022).

The GC-MS phytochemical analysis of *T. minuta* essential oil revealed the main constituents to be cis-ocimen, beta-ocimen, rosefuran, limonene, dihydrotagetone, trans-tagetone, cis tageton, cistagetonone, tagetone (Z)-ocimenone and (E)-ocimensone which have been documented to have antimicrobial activity (Yeasmin & Gupta, 2022).

Four flavonols have been identified in extracts of *Tagetes minuta* include 6-hydroxyquercetin 7-O-β-(6-galloylglucopyranoside), 6-hydroxykaempferol (7-O-β-glucopyranoside), 6-hydroxykaempferol 7-O-β-(6-galloylglucopyranoside) and 6-hydroxyquercetin 7-O-β-(6-caffeoylglucopyranoside) (Shahzadi & Shah, 2015). The authors reported flavonols suchaspatuletin, quercetin, quercetagetin and isorhamnetin along with some of their glycosides had previously been detected in *T. minuta*.

In addition to lesser quantities of sesquiterpene hydrocarbons and oxygenated compounds, the essential oils of *Tagetes minuta* are generally rich in monoterpene hydrocarbons (ocimenes, limonene, terpinene, myrcene) bicyclic and acyclic monoterpene ketones

(tagetone, dihydrotagetone and tagetenone), which are the main odorants (Salehi *et al.*, 2018). Sadia *et al.,* (2015) reported similar findings.

Thirteen (13) compounds, including dihydrotagetone, artemisia, (Z)-tagetenone, (-)-spathulenol and estragole were identified in the essential oil of *Tagetes minuta* L. by Abdoul-Latif *et al.,* (2022). Akram & Tembhre, (2016) reported that extracts of *Tagetes minuta* contained alkaloids, terpenoids, flavonoids, saponins, glycosides and tannins. Rikisahedew *et al.,* (2023) and Opinde *et al.,* (2018) also reported similar findings from phytochemical analyses of *Tagetes minuta* extracts.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample collection and storage

Plant leaves of *Achyranthes aspera* and *Tagetes minuta* used in this study were identified and then collected from the farm fields of University of Eldoret in the months of January and February 2024. *Achyranthes aspera* and *Tagetes minuta* both grow as weeds in uncultivated farm land areas particularly to those that have been abandoned. The samples were placed in sterile sample collection bags, assigned codes AA for *Achyranthes aspera* and TM for *Tagetes minuta*. They were placed in sterile sample collection containers and then transported to University of Eldoret Biotechnology laboratory.

3.2 Preparation of plant samples

After collection, the plant leaves were hand washed in running tap water two times. Washing eliminated contamination from adhering particles such as any dirt particles present on the leaves surface. The washed plants were then dried to remove the water content from plants. The plants were weighed then placed on stands to be air-dried under a shed to ensure that the plant's active compounds are not lost (Balamurugan *et al.*, 2019). This was done while weighing the samples daily until there was no more weight change. After complete drying, the samples were ground well using pestle and mortar, weighed, labeled and then stored in air tight sample collection containers ready for further analysis.

3.3 Extraction of the plant extracts

The powder from the plant leaves were each used for crude extraction. Water and ethanol were used as solvents. One gram of sample to 5 ml of solvent (1:5) was the ratio utilised in the extraction process. The extracted solutions were filtered by passing the extracts through

Whatman No. 1 filter paper, collected and then concentrated using a rotary evaporator (Model: RE-300) at 65°C. One (1) g of the crude extract was dissolved into 1 millilitre of the respective solvents to make stock concentrations of 1000 mg/ml. The percentage yield was calculated using the formula below by Felhi *et al.* (2017);

$$\% \text{ yield} = \frac{\text{Weight of extract obtained after evaporation of solvent}}{\text{Dry weight of the plant sample}} \times 100$$

3.3.1 Aqueous extraction

Thirty (30) grams each of *Achyranthes aspera* and *Tagetes minuta* crushed leaf powders were weighed and soaked in 150 ml double distilled water in each case. The mixtures were then shaken ferociously before being placed for two hours in a shaking water bath at a temperature of 50°C. They were then left to settle with continuous agitation in a shaker at room temperature for 48 hours. Mixtures were removed and filtered using Whatman No. 1 filter papers. The filtrates were then transferred into round bottom flasks then placed in a water bath to concentrate. The concentrated samples were covered using a sterile cotton wool and an aluminium foil then stored in a fridge set at 4°C awaiting antibacterial assays, allelopathy testing and phytochemical screening.

3.3.2 Extraction using Ethanol

Thirty (30) grams each of *Achyranthes aspera* and *Tagetes minuta* crushed leaf powders were weighed, placed into separate 250ml conical flask and 150 ml of ethanol was added to each sample then shaken well. In each case, the mixtures were allowed to macerate with continuous agitation in a shaker for 48 hours. They were then filtered using Whatman's no. 1 filter papers and the filtrates concentrated using a rotary evaporator. The concentrated pastes were sealed and stored in a fridge set at 4°C awaiting subsequent processes.

3.4 Antibacterial bioassays of *Achyranthes aspera* and *Tagetes minuta* leaf extracts.

3.4.1 Culture media preparation

Nutrient agar was used for bioassays. Twenty-eight (28) grams of nutrient agar was suspended in 1000 ml of distilled water. Media was sterilized at 121^{o}C for 15 minutes in an autoclave (Model: LS-50LJ), cooled to 40^{o}C before dispensing 15-20 ml into sterile petri plates and allowed to further solidify.

3.4.2 Microorganisms used and their preparation

American Type Culture Collection (ATCC) bacteria (*Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 51299, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213) obtained from KEMRI were used in the study. The ATCC bacteria were sub-cultured onto nutrient agar and then incubated at 37^{o}C for 24 hours to obtain freshly grown bacterial cultures. The freshly obtained cultures were then inoculated into nutrient broth in sterile bottle, labelled then stored in a fridge set at 4°C awaiting subsequent bioassays.

3.4.3 Susceptibility testing

In accordance with recommendations from the Clinical and Laboratory Standards Institute's (CLSI, 2022) susceptibility tests were carried out utilizing the disc diffusion method. Disc diffusion was performed using nutrient agar media. The microbial cultures were inoculated using a sterile swab by distributing the cultures uniformly across the entire surface of the media. Six (6) mm filter paper discs made from Whatman No. 1 filter paper with 0.1 ml of extracts with different concentrations (10^{0} to 10^{-2}) was micro pipetted into the sterile filter paper discs and allowed to soak. Ciprofloxacin was utilised as a positive

control with the corresponding solvents used as negative controls. Discs with desired concentrations were aseptically placed onto plates containing media and inoculated using a sterile forceps before and then incubated at 37°C for 24 hrs. All experiments were conducted in three replicates in a completely randomised design (CRD).

Clear zones which formed around the disc were measured from edge to edge as zones of inhibition. Inhibition zone diameters were measured in mm, recorded and interpreted as per the Clinical and Laboratory Standards Institute (CLSI), 2022 guidelines.

3.4.4 Data handling and statistical analysis for antibacterial activity.

Antibacterial bioactivity of test extracts against *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 51299, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853 as well as *Staphylococcus aureus* ATCC 29213 was evaluated using disk diffusion where the inhibition zone diameters were measured in millimeters and entered into Microsoft Excel 2021 and the data obtained was entered in separate spreadsheets. Means were calculated and expressed as means ± standard error then presented using bar graphs. The relationships between the plants used, concentration of the extract, the test microorganism as well as the solvents used for extraction at 95% confidence level was determined using Analysis of variance (ANOVA). Statgraphics centurion software version XVI was used for analysis.

3.5 Allelopathic activities of *Achyranthes aspera* and *Tagetes minuta* leaf extracts.

3.5.1 Testing for allelopathic activity.

Thirty grams each of *Achyranthes aspera* and *Tagetes minuta* crushed leaf powders were soaked in 100 ml double distilled water and ethanol in separate conical flasks for 48 hrs.

Dilutions of extracts with double distilled water was prepared for concentrations of 10^0 to 10^{-3}. Effects of the various extracts on germination was tested by placing seeds of maize (H6213), millet (pearl millet), rice (NERICA L-19) and sorghum (E-1291) obtained from Kenya Seed Company in petri dishes.

The seeds used for the germination test were surface sterilized, first, by soaking in 1% sodium hypochlorite (NaOCl) for 1 min and rinsing two times in double distilled water to remove excess chemicals. The petri dishes were cleaned, autoclaved and then had sterilized serviettes put on the bottom as a thin lining.

Germination assay of the plant extracts were conducted by placing 5 surface sterilized seeds in each petri dish. Thirty millilitres of the test extracts from the experimental plants were then added to it. Additionally, to assess the potential for synergy, 15 ml of the extracts of each of the treatments were mixed and dispensed into separate petri dishes.

The petri dishes treated with distilled water was set as positive control with those with Dimethyl sulfoxide set as negative control. The design of the experiments was Completely Randomized with three replications for each plant extracts and test seed species. The setup was then kept undisturbed at room temperature (25±2°C) in the laboratory.

The emerged plumule and radicle lengths were measured after 7 days using a sterile string, transferred onto a ruler the measurements recorded. The number of seeds germinated was also counted after 7 days and the germination percentages calculated using the formula below:

$$\text{Germination percentage (\%)} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \times 100$$

3.5.2 Data handling and statistical analysis for allelopathic activity.

A database was created in Microsoft Excel 2021 where data was entered in separate spreadsheets. The effect of the test plant extracts on seed germination, plumule and radicle lengths was determined using analysis of variance (ANOVA) and considered statistically significant at 95% confidence level ($P \leq 0.05$). All analysis was conducted using Statgraphics centurion software version XVI. Tukey's Honestly Significant Difference (HSD) was used to separate means.

3.6 Phytochemical composition of *Achyranthes aspera* and *Tagetes minuta* leaf extracts.

For qualitative phytochemical analysis, the already prepared stock concentrations were utilised. The aqueous and ethanolic filtrates were used to screen for the absence or presence of the different phytochemicals according to the following described protocols;

3.6.1 Test for alkaloid

In a test tube, one millilitre of extract was mixed with one millilitre of 1% HCl. This was followed by addition of 3-5 drops of Wagner's reagent (2 g of iodine and 6 g of potassium iodide in 100 ml distilled water). Formation of a reddish-brown coloured precipitate indicated presence of alkaloids (Balamurugan *et al.,* 2019).

3.6.2 Test for anthraquinones

In a test tube, one millilitre of extract was mixed with three millilitres of dilute ammonia. After which 2-3 drops of concentrated sulphuric acid was added to the mixture. The rose-pink appearance in the mixture indicated presence of anthraquinones (Balamurugan *et al.,* 2019).

3.6.3 Test for coumarins

Two millilitres of each sample was added to three millilitres of 10% sodium hydroxide in a test tube and shaken well for a minute. Formation of yellow colour indicated presence of coumarins (Balamurugan *et al.*, 2019).

3.6.4 Test for flavonoids

A 5 ml sample of a dilute ammonia solution was put into a test tube with 1 mL of extract, and it was shaken well. The aqueous portion formed was separated using a pipette then the introduction of three drops of concentrated sulphuric acid. Presence of flavonoids was indicated by the emergence of yellow colour (Balamurugan *et al.*, 2019).

3.6.5 Test for glycosides

Two ml of plant extract was added to three mL of chloroform in a tube and mixed. The chloroform layer was removed by pipette and 10% ammonia was then added. Presence of glycosides was confirmed by appearance of a pink colour (Balamurugan *et al.*, 2019).

3.6.6 Test for phenols

Five millilitres of the extract were combined gently with three millilitres of a 10% lead acetate solution. Presence of phenols was detected by the production of a large white precipitate (Balamurugan *et al.*, 2019).

3.6.7 Test for quinones

One millilitre of alcoholic potassium hydroxide (KOH) was added to 2 ml of extract in a test tube and shaken well. Presence of red to blue colour was indicative that quinones were present in the sample (Balamurugan *et al.*, 2019).

3.6.8 Test for steroids

Two ml of chloroform was added to 2 ml of extract in a clean/test tube. After adding 2 ml of concentrated sulfuric acid the mixture was then agitated. The appearance of red colour and yellowish green fluorescence showed that steroids were present in that sample (Balamurugan *et al.*, 2019).

3.6.9 Test for saponins

In a test tube, 2 millilitres of extract was drawn then mixed thoroughly with 2 millilitres of distilled water then shaken vigorously and warmed up. A few drops of olive oil were then introduced, shaken vigorously and then observed if there is formation of emulsion which was indicative that saponins were present (Bibi *et al.,* 2016; Balamurugan *et al.,* 2019).

3.6.10 Test for tannins

To detect presence of tannins, five millilitres of the filtered plant extract was transferred into a sterile test tube, followed by the gentle addition of three drops of a 5% ferric chloride solution. The appearance of a deep green coloration indicated the presence of tannins in the sample (Balamurugan *et al.,* 2019).

3.6.11 Test for terpenoids

In a test tube, 1 ml of chloroform was mixed with 2 ml of the sample. To this mixture, 3 drops of concentrated sulphuric acid was added to the mixture through the side of the test tube in order to form a layer. The appearance of a reddish-brown at the interface indicated that terpenoids were present in the sample (Balamurugan *et al.*, 2019).

CHAPTER FOUR

RESULTS

4.1 Percentage yields of leaf extracts

After concentration of extracts, *Achyranthes aspera* and *Tagetes minuta* water and ethanolic extracts were weighed and the percentage yield was calculated as described by Felhi *et al.* (2017). The mean percentage yield after extraction for *Achyranthes aspera* was 21.16% with *Tagetes minuta* having a mean percentage yield of 21.77% (Table 4.1). Aqueous extracts from *Achyranthes aspera* had the highest percentage yield at 34.29% with the lowest percentage yield being from ethanolic extracts from the same plant. Ethanolic and aqueous leaf extracts of *Tagetes minuta* had a percentage yield of 20.46% and 23.08% respectively (Table 4.1).

Table 4.1: Percentage yields after extraction

Plant	Solvent	% Yield of leaf extracts	Mean % yield
Achyranthes aspera	Ethanol	8.03	21.16
	Water	34.29	
Tagetes minuta	Ethanol	20.46	21.77
	Water	23.08	

4.2 Efficiency of solvents used for extraction

Solvents used during the extraction process were water and ethanol. The efficiency of each of the two solvents was different as evident from percentage yields obtained after extraction (Table 4.1). The crude extracts obtained from water had a higher mean percentage yield of 28.68% when compared to that of ethanol which yielded mean percentage yield of 14.25% (Figure 4.1).

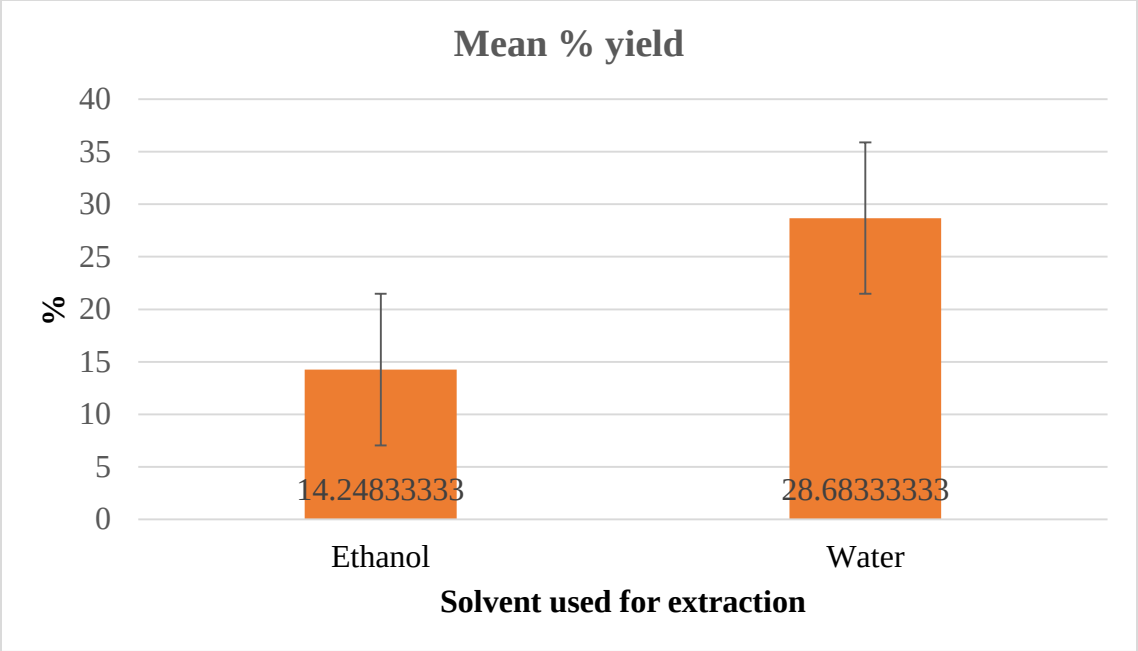

Figure 4.1: Solvents utilised in the extraction process and their mean percentage yield.

4.3 Antibacterial activities of *Achyranthes aspera* and *Tagetes minuta* crude extracts

The crude leaf extracts of *Achyranthes aspera* and *Tagetes minuta* exerted antibacterial activity to the test bacteria variably. *Tagetes minuta* had a higher antibacterial activity on *Staphylococcus aureus* ATCC 29213 while *Achyranthes aspera* had a higher antibacterial activity on the other test bacteria when the activity of extracts from both plants was compared. On average, Ethanolic extracts had a higher antibacterial activity in comparison to aqueous extracts. The extracts obtained were active at 10^{0} and 10^{-1} in all the test bacteria with, only *Staphylococcus aureus* ATCC 29213 was inhibited by the extracts at 10^{-2}. However, the extract(s) bioactivity was inferior to that of ciprofloxacin.

The bioactivity of extracts in all the concentrations used (10^{0} to 10^{-2}) on average was greater against *Staphylococcus aureus* ATCC 29213 followed by *Enterococcus faecalis* ATCC 51299 then *Escherichia coli* ATCC 25922 then *Pseudomonas aeruginosa* ATCC 27853 with little activity observed on *Klebsiella pneumoniae* ATCC 700603.

The zones of inhibition varied depending on the test bacteria, extract concentration, the plant used and/or the solvent used for extraction. The zones of inhibition decreased with decreasing extract's concentration with 10^0 having bigger zones compared to 10^{-1} and 10^{-2} concentrations. Plate 4.1 a & b shows the bioactivity of *Achyranthes aspera* and *Tagetes minuta* extracts against *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 respectively.

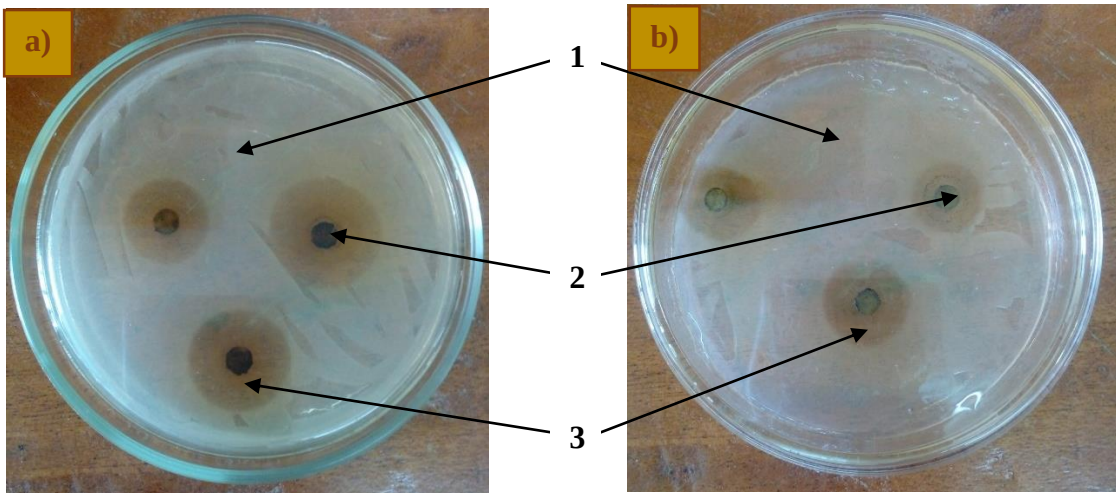

Plate 4.1: *E. coli* on *Achyranthes aspera* extract (a) and *K. pneumoniae* on *Tagetes minuta* extract (b).

Legend: 1 – Bacteria, **2** – 6mm discs impregnated with extract, **3** – Zones of inhibition

The results obtained were analyzed using Analysis of variance (ANOVA) whereby the p-values ranged 0.0000 to 0.9213 at 95.0% confidence level ($P \leq 0.05$). Twelve (12) out of the fifteen p-values were less than 0.05 meaning these factors (concentration of the extract, solvent used, plant species and most of the interactions) had a statistically significant effect on the zones of inhibition (diameters in mm) at $P \leq 0.05$ (Appendix II). This suggests that the extracts of *Achyranthes aspera* and *Tagetes minuta* as evident from the results obtained in this study can be used as antibacterial agents.

4.3.1 Bioactivity of extracts against *Escherichia coli* ATCC 25922

Effects of *Achyranthes aspera* and *Tagetes minuta* aqueous and ethanolic extracts on *Escherichia coli* ATCC 25922 (mean zones of inhibition with standard error bars) is shown in Figure 4.2. The mean zone of inhibition for *E. coli* ATCC 25922 at 10^0 was 25.5mm and 22.5mm for *A. aspera* and *T. minuta* respectively. Aqueous extracts were more detrimental with a mean inhibition zone diameters of 28mm and 27mm when compared to 22mm and 18mm from ethanolic extracts *A. aspera* and *T. minuta* respectively. Reducing extract effectiveness with reducing concentration was noted as shown in Figure 4.2.

ANOVA was used to analyze for statistical significance of the effect on *Escherichia coli* ATCC 25922 which returned p-values of 0.0000 for both *Achyranthes aspera* and *Tagetes minuta* at $P \leq 0.05$ (Table 4.2 & Table 4.3). That means that the extracts of both plants had a statistically significant effect on the zone of inhibitions of *Escherichia coli* ATCC 25922.

Table 4.2: ANOVA table on effects of *Achyranthes aspera* aqueous and ethanolic extracts on Inhibition zone diameters of *Escherichia coli* ATCC 25922.

Source of Variation	SS	Df	MS	F	P-value	Inference
Between Groups	1290.944	5	258.1889	34.4252	0.0000	Significant
Within Groups	90	12	7.5			
Total	1380.944	17				

Table 4.3: ANOVA table on effects of *Tagetes minuta* aqueous and ethanolic extracts on Inhibition zone diameters of *Escherichia coli* ATCC 25922.

Source of Variation	SS	df	MS	F	P-value	Inference
Between Groups	949.1667	5	189.8333	30.2389	0.0000	Significant
Within Groups	75.3333	12	6.277778			
Total	1024.5	17				

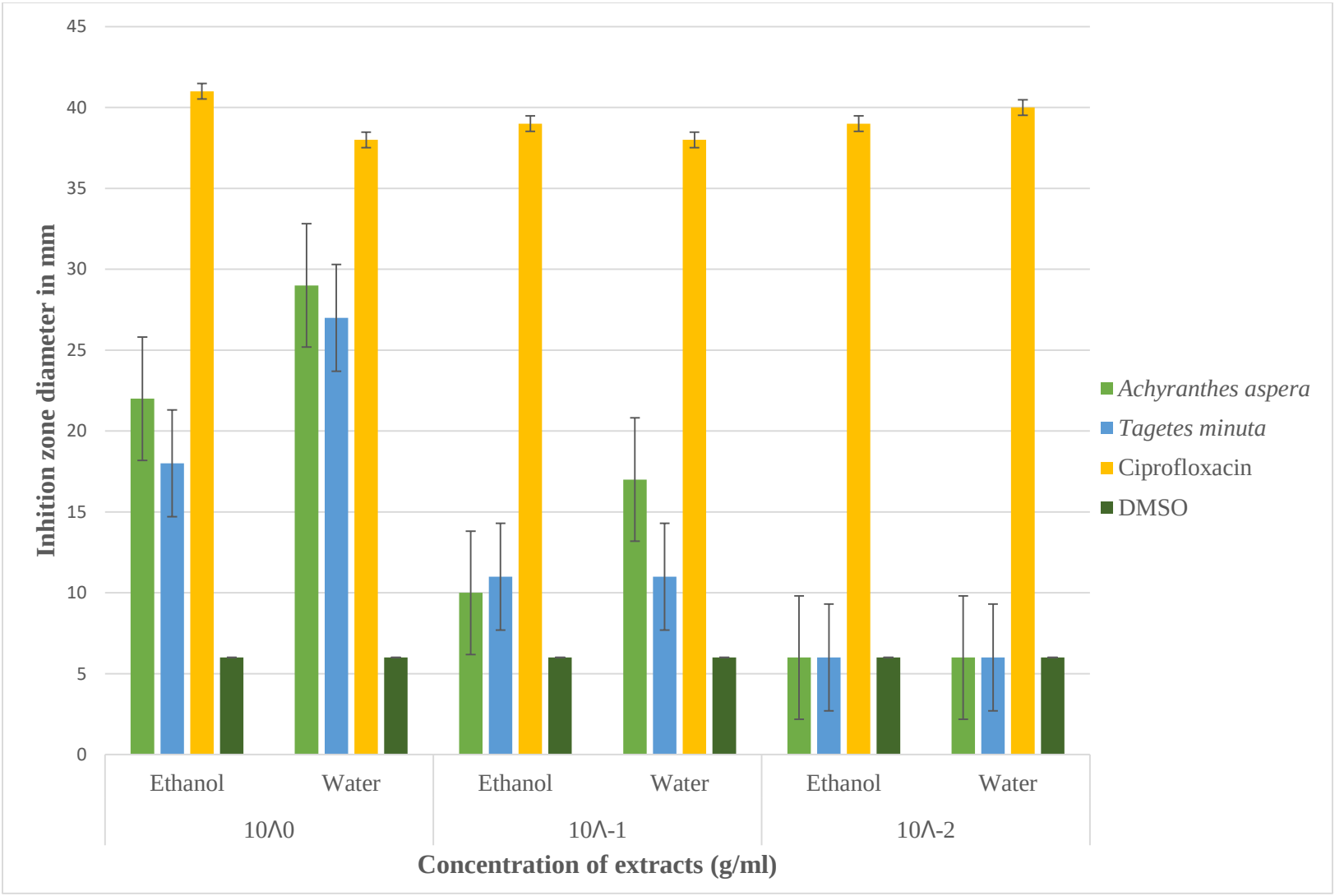

Figure 4.2: Antibacterial activities of *Achyranthes aspera* and *Tagetes minuta* against *Escherichia coli* ATCC 25922.

4.3.2 Bioactivity of extracts against *Enterococcus faecalis* ATCC 51299

Figure 4.3 illustrates the effects of ethanolic and aqueous extracts from *Achyranthes aspera* and *Tagetes minuta* on *Enterococcus faecalis* ATCC 51299 (mean zones of inhibition with standard error bars) as well as both positive and negative controls. The average inhibition zones for *T. minuta* and *A. aspera* at 10^0 was 25.5 mm. Compared to aqueous extracts, which had a mean zone of inhibition of 26mm and 27mm, ethanolic extracts were less more harmful with inhibition zones of 25mm and 24mm for *A. aspera* and *T. minuta* respectively. Reducing extract effectiveness with reducing concentration was also observed (Figure 4.3).

Using ANOVA, the effect on *Enterococcus faecalis* ATCC 51299 was analyzed for statistical significance. The results showed that both *Achyranthes aspera* and *Tagetes minuta* had p-values of 0.0000 at 95.0% confidence level ($P < 0.05$) (Table 4.4 & Table 4.5). This indicates that *Enterococcus faecalis* ATCC 51299's zone of inhibition was significantly affected by the extracts from both plants.

Table 4.4: ANOVA table on effects of *Achyranthes aspera* aqueous and ethanolic extracts on Inhibition zone diameters of *Enterococcus faecalis* ATCC 51299.

Source of Variation	SS	df	MS	F	P-value	Inference
Between Groups	1236.444	5	247.2889	35.6096	0.0000	Significant
Within Groups	83.3333	12	6.9444			
Total	1319.778	17				

Table 4.5: ANOVA table on effects of *Tagetes minuta* aqueous and ethanolic extracts on Inhibition zone diameters of *Enterococcus faecalis* ATCC 51299.

Source of Variation	SS	df	MS	F	P-value	Inference
Between Groups	1266.667	5	253.3333	57	0.0000	Significant
Within Groups	53.3333	12	4.4444			
Total	1320	17				

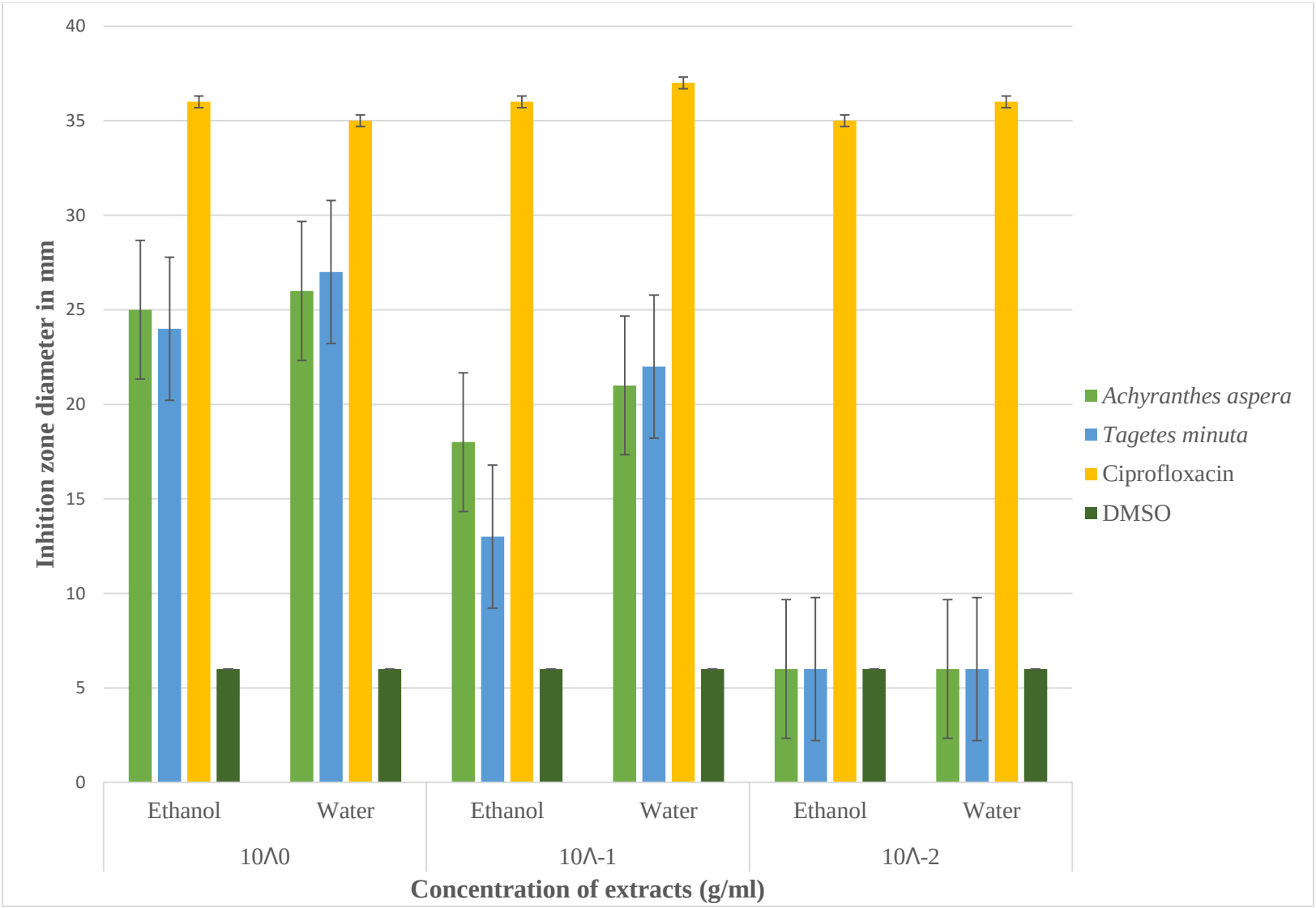

Figure 4.3: Antibacterial activities of *Achyranthes aspera* and *Tagetes minuta* against *Enterococcus faecalis* ATCC 51299.

4.3.3 Bioactivity of extracts against *Klebsiella pneumoniae* ATCC 700603

Effects of *Achyranthes aspera* and *Tagetes minuta* aqueous and ethanolic leaf extracts on *K. pneumoniae* ATCC 700603 (mean zones of inhibition with standard error bars) is shown in Figure 4.4. The mean zone of inhibition for *K. pneumoniae* at 10^0 was 15mm and 12.5mm for *A. aspera* and *T. minuta* respectively. Extracts of *A. aspera* were more detrimental with a mean zone of inhibition of 14mm and 16mm from aqueous and ethanolic extracts when compared to 12mm and 13mm from *T. minuta*. Reducing extract effectiveness with reducing concentration was noted as shown in Figure 4.4.

ANOVA was used in analyzing for statistical significance of the effect on *K. pneumoniae* ATCC 700603 which returned p-values of 0.0003 for *A. aspera* and 0.0109 for *T. minuta* at $P \leq 0.05$ (Table 4.6 & Table 4.7). That means that the extracts of both plants had a statistically significant effect on the zone of inhibitions of *K. pneumoniae* ATCC 700603.

Table 4.6: ANOVA table on effects of *Achyranthes aspera* aqueous and ethanolic extracts on Inhibition zone diameters of *Klebsiella pneumoniae* ATCC 700603.

Source of Variation	SS	df	MS	F	P-value	Inference
Between Groups	296	5	59.2	11.4581	0.0003	Significant
Within Groups	62	12	5.1667			
Total	358	17				

Table 4.7: ANOVA table on effects of *Tagetes minuta* aqueous and ethanolic extracts on Inhibition zone diameters of *Klebsiella pneumoniae* ATCC 700603.

Source of Variation	SS	df	MS	F	P-value	Inference
Between Groups	145.6111	5	29.1222	4.9453	0.0109	Significant
Within Groups	70.6667	12	5.8889			
Total	216.2778	17				

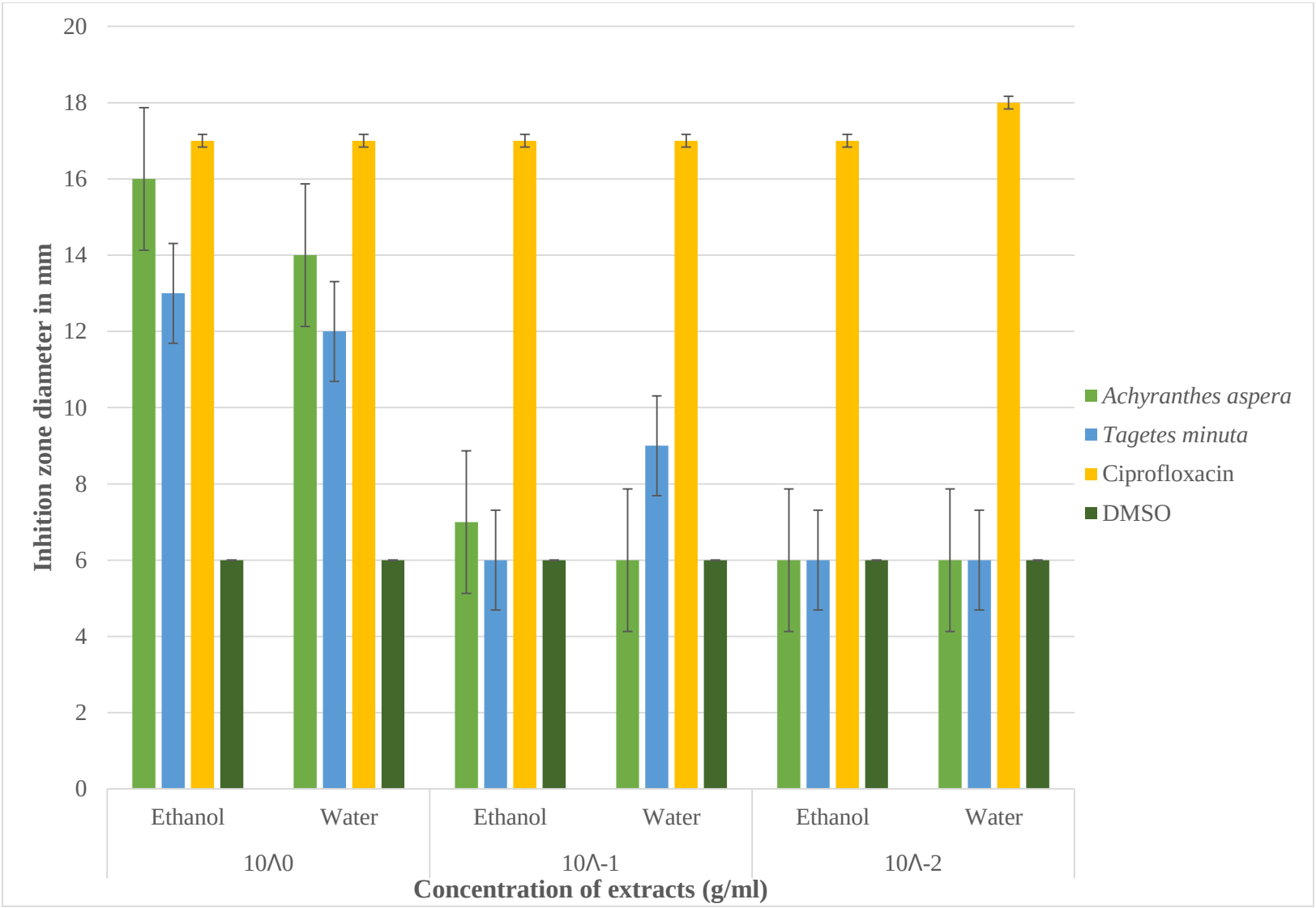

Figure 4.4: Antibacterial activities of *Achyranthes aspera* and *Tagetes minuta* against *Klebsiella pneumoniae* ATCC 700603.

4.3.4 Bioactivity of extracts against *Pseudomonas aeruginosa* ATCC 27853

Figure 4.5 illustrates the effects of ethanolic and aqueous leaf extracts from *Achyranthes aspera* and *Tagetes minuta* on *Pseudomonas aeruginosa* ATCC 27853 (mean zones of inhibition with standard error bars) as well as both the positive and negative controls. The average zone of inhibition for *T. minuta* and *A. aspera* at 10^0 was 26mm and 24mm. The mean zone of inhibition for ethanolic extracts was 27.5mm, which was greater than the 22.5mm for aqueous extracts at 10^0. Reducing extract effectiveness with reducing concentration was also documented as displayed in Figure 4.5.

Using ANOVA, the effect on *P. aeruginosa* ATCC 27853 was analysed for statistical significance. The results showed that both *A. aspera* and *T. minuta* had p-values of 0.0000 at $P < 0.05$ (Table 4.8 & Table 4.9). This indicates that *P. aeruginosa* ATCC 27853's zone of inhibition was significantly affected by leaf extracts of both plants.

Table 4.8: ANOVA table on effects of *Achyranthes aspera* aqueous and ethanolic extracts on Inhibition zone diameters of *Pseudomonas aeruginosa* ATCC 27853.

Source of Variation	SS	df	MS	F	P-value	Inference
Between Groups	1224.278	5	244.8556	65.7821	0.0000	Significant
Within Groups	44.6667	12	3.7222			
Total	1268.944	17				

Table 4.9: ANOVA table on effects of *Tagetes minuta* aqueous and ethanolic extracts on Inhibition zone diameters of *Pseudomonas aeruginosa* ATCC 27853.

Source of Variation	SS	df	MS	F	P-value	Inference
Between Groups	1642.667	5	328.5333	31.4553	0.0000	Significant
Within Groups	125.3333	12	10.4444			
Total	1768	17				

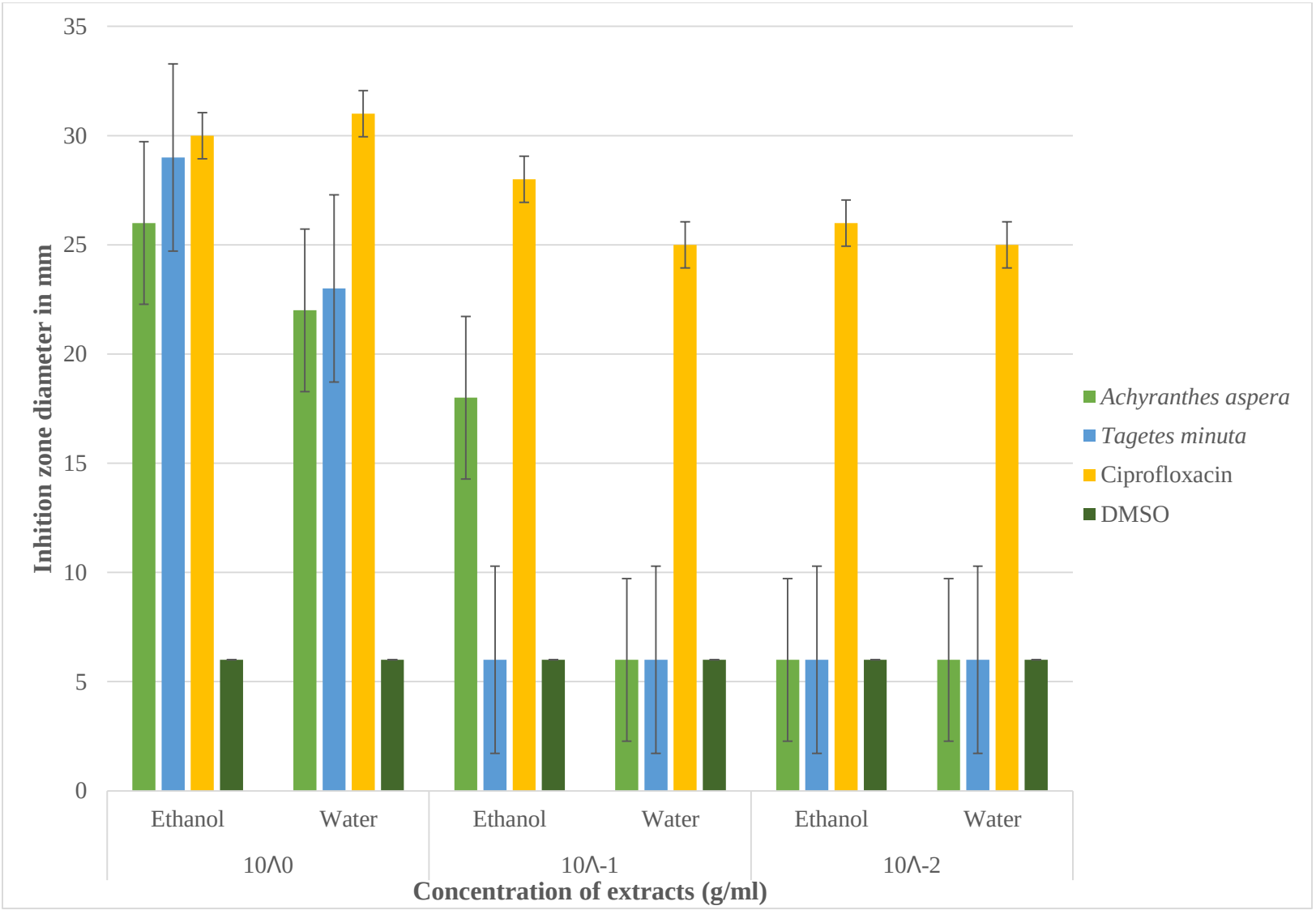

Figure 4.5: Antibacterial activities of *Achyranthes aspera* and *Tagetes minuta* against *Pseudomonas aeruginosa* ATCC 27853.

4.3.5 Bioactivity of extracts against *Staphylococcus aureus* ATCC 29213

Effects of *Achyranthes aspera* and *Tagetes minuta* ethanolic and aqueous leaf extracts on *S. aureus* ATCC 29213 (mean zones of inhibition with standard error bars) is shown in Figure 4.6. The mean zone of inhibition for *S. aureus* at 10^0 was 38mm and 40mm for *A. aspera* and *T. minuta* respectively. Ethanolic extracts were more detrimental with a mean zone of inhibition of 41mm when compared to 37mm from aqueous extracts at 10^0. Reducing extract effectiveness with reducing concentration was also reported (Figure 4.6).

ANOVA was utilized in analyzing for statistical significance on the effect of extracts on *Staphylococcus aureus* ATCC 29213 which returned p-values of 0.0041 for *Achyranthes aspera* and 0.0193 for *Tagetes minuta* at 95.0% confidence level ($P \leq 0.05$) (Table 4.10 & Table 4.11). That means that the extracts from both plants had a statistically significant effect on the zone of inhibitions of *Staphylococcus aureus* ATCC 29213.

Table 4.10: ANOVA table on effects of *Achyranthes aspera* aqueous and ethanolic extracts on Inhibition zone diameters of *Staphylococcus aureus* ATCC 29213.

Source of Variation	SS	df	MS	F	P-value	Inference
Between Groups	523.1111	5	104.6222	6.3837	0.0041	Significant
Within Groups	196.6667	12	16.3889			
Total	719.7778	17				

Table 4.11: ANOVA table on effects of *Tagetes minuta* aqueous and ethanolic extracts on Inhibition zone diameters of *Staphylococcus aureus* ATCC 29213.

Source of Variation	SS	df	MS	F	P-value	Inference
Between Groups	355.3333	5	71.0667	4.2079	0.0193	Significant
Within Groups	202.6667	12	16.8889			
Total	558	17				

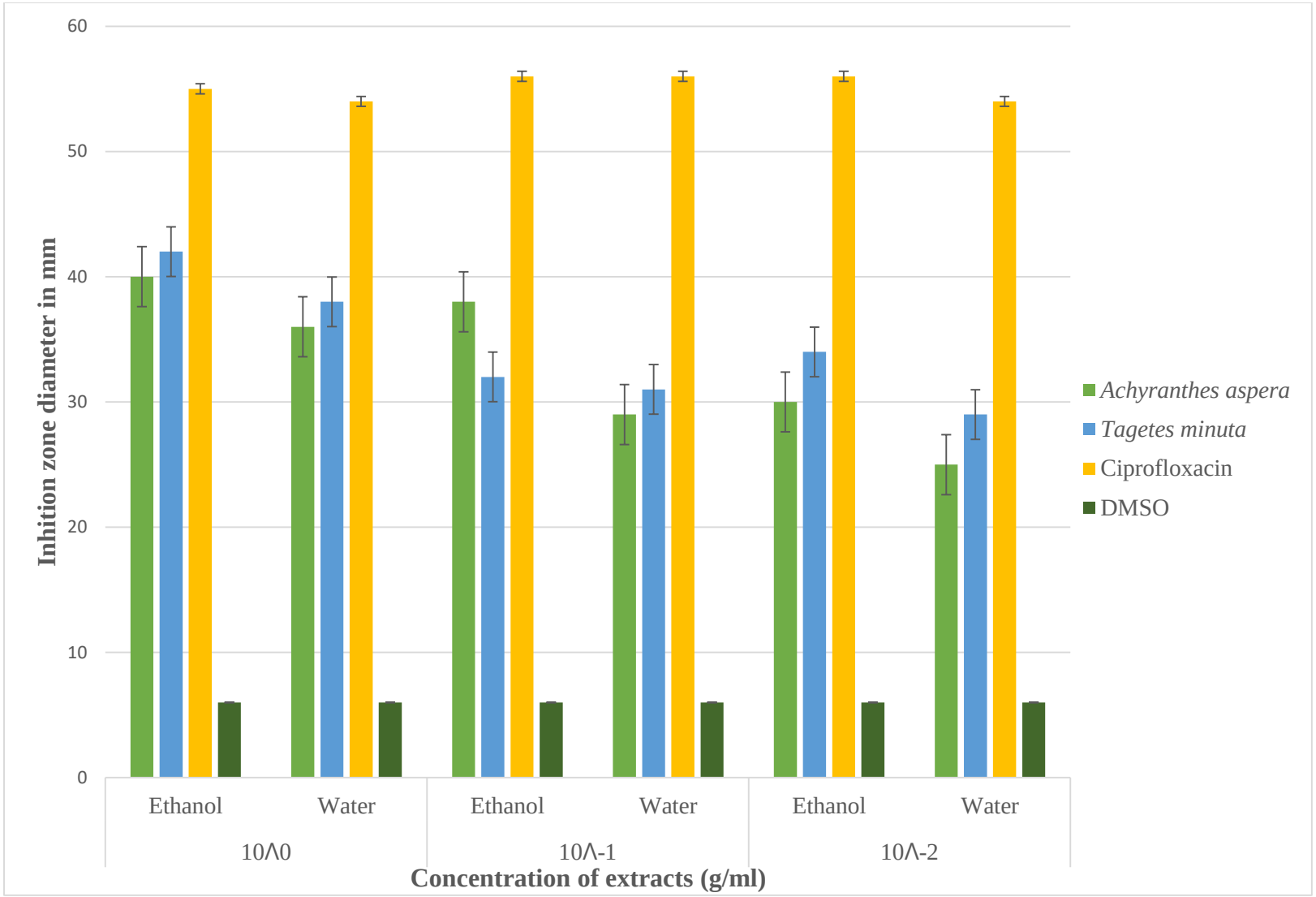

Figure 4.6: Antibacterial activities of *Achyranthes aspera* and *Tagetes minuta* against *Staphylococcus aureus* ATCC 29213.

4.4 Effect of *Achyranthes aspera* and *Tagetes minuta* crude plant extracts on Seed germination

Allelopathic effects of *Achyranthes aspera* and *Tagetes minuta* crude leaf extracts on seed germination was tested on seeds of maize, millet, rice and sorghum in petri dishes. Aqueous and ethanolic extracts were evaluated with ethanolic extracts showing a relatively higher activity in comparison to aqueous based extracts. On average, the effects of these extracts was most pronounced in rice followed by maize then sorghum with minimal effect on millet. Overall, the radicle lengths were more adversely affected than the plumule lengths. A gradual decline in the allelopathic effect was observed with decreasing extract concentration which indicated that higher concentrations produced stronger inhibitory effects. This meant the effect determined from their percentage reductions was greatest at concentration 10^0 when compared to the diluted concentrations of 10^{-1}, 10^{-2} and 10^{-3}.

4.4.1 Germination percentages.

The number of seeds germinated was counted after 7 days so as to calculate the germination percentages. There was increased germinated seed with reduced extract concentration. In general, Millet had the highest germination percentage of 89.44% while rice had the lowest at 67.78%. Maize and sorghum had 87.50% and 85.83% respectively (Table 4.12).

Table 4.12: Number of seeds germinated after 7 days and germination percentages

Plant	*Achyranthes aspera* (A)	*Tagetes minuta* (T)	A+T	Total	Germination %	% of +ve Control	% of -ve Control
Maize	102	109	104	315	87.50	100	0
Millet	100	107	115	322	89.44	100	0
Rice	75	88	81	244	67.78	98	0
Sorghum	96	109	104	309	85.83	100	0

4.4.2 Effects of ethanolic and aqueous leaf extracts of *Achyranthes aspera* and *Tagetes minuta* on plumule lengths

a) Maize

The highest percentage reduction in plumule lengths of maize was on aqueous extracts of *Tagetes minuta* (83%) with the lowest reduction being on aqueous extracts of *Achyranthes aspera* (65%) based concentration 10^0 (Figure 4.7). Ethanolic extracts of *Achyranthes aspera* and *Tagetes minuta* had percentage reductions of 68% and 71% respectively. A combination of the two extracts resulted in reductions of 71% and 76% for ethanolic and aqueous extracts respectively. The other extract concentrations showed a similar pattern of results. However, reduction in inhibitory activity with reducing extract concentrations was observed as illustrated by Figure 4.7 below.

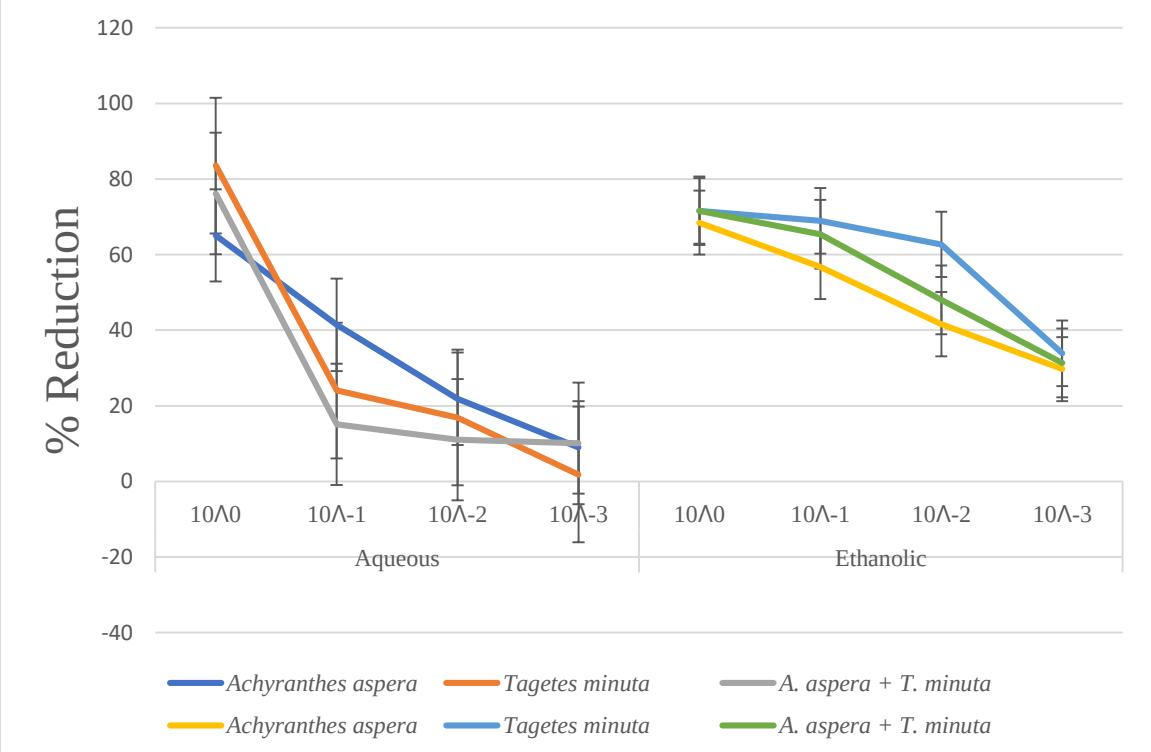

Figure 4.7: Percentage reductions in plumule lengths of Maize exposed to plant extract(s).

b) Millet

The plumule lengths of millet were affected differently with the original concentration (10^0) being more detrimental. The effect was then observed to be reducing with reducing extracts concentrations. Ethanolic extracts of *Achyranthes aspera* produced the highest percentage reduction (90%) with the lowest percentage reduction being of a combination of aqueous extracts obtained from both plants (69%) (Figure 4.8). Ethanolic and aqueous extracts from *Tagetes minuta* had percentage reductions of 84% and 89% respectively at concentration 10^0 (Figure 4.8).

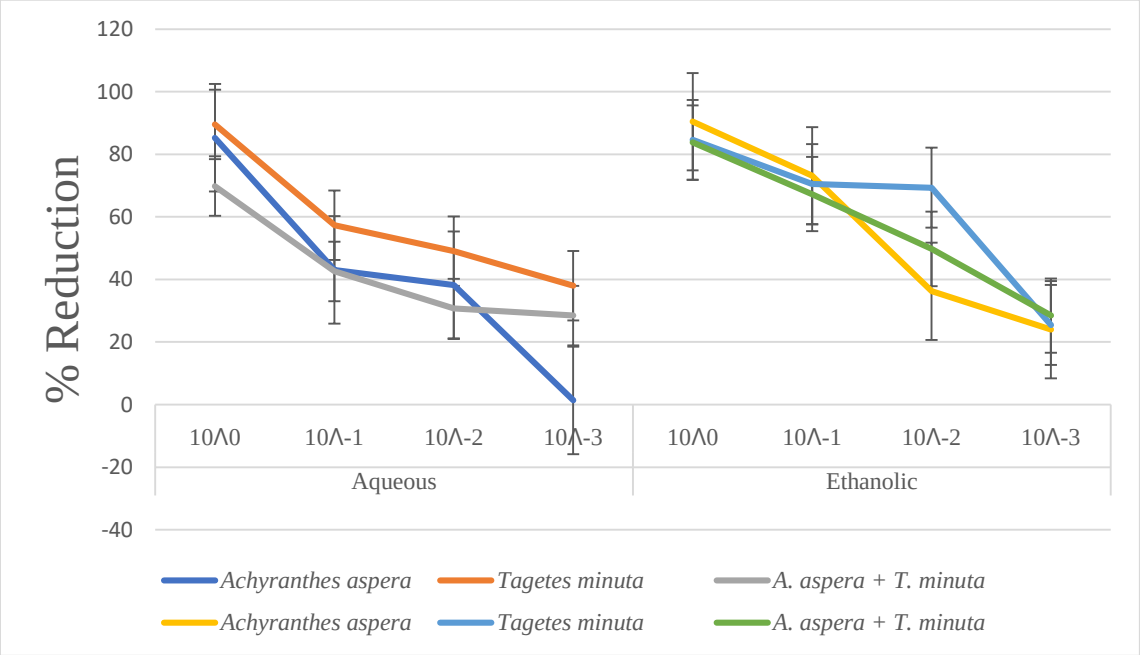

Figure 4.8: Percentage reductions in plumule lengths of Millet exposed to plant extract(s).

c) Rice

The plumule lengths of rice were adversely affected by the extracts from *Achyranthes aspera* and *Tagetes minuta* as well as extracts from a combination of these two plants. This was evident from the percentage reductions of more than 86% in all the tested extracts based on the undiluted concentration (10^0). All the aqueous extracts had percentage

reductions of more than 90%. There was reducing effect with reducing extract concentrations, from 10^0 towards 10^{-3}, observed as well (Figures 4.9).

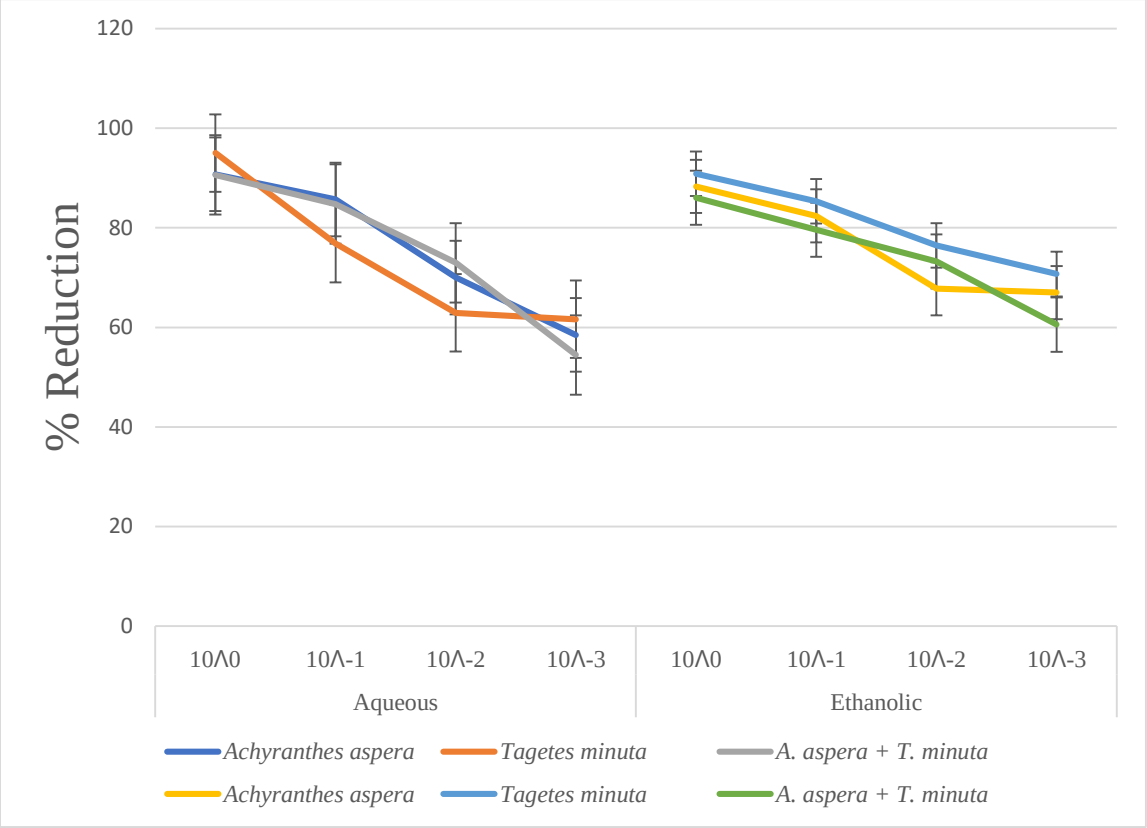

Figure 4.9: Percentage reductions in plumule lengths of Rice exposed to plant extract(s).

d) Sorghum

The highest percentage reduction in plumule lengths of sorghum was on ethanolic extracts of *Tagetes minuta* (92%) with the lowest reduction being on aqueous extracts from the same plant (63%) according to concentration 10^0 (Figure 4.10). Ethanolic and aqueous extracts obtained from *Achyranthes aspera* had percentage reductions of 72% and 83% respectively. A combination of the two extracts resulted in reductions of 81% and 74% for ethanolic and aqueous extracts respectively. The other extract concentrations followed a similar pattern. However, reducing activity with reducing extract concentrations was observed as illustrated by Figure 4.10 below.

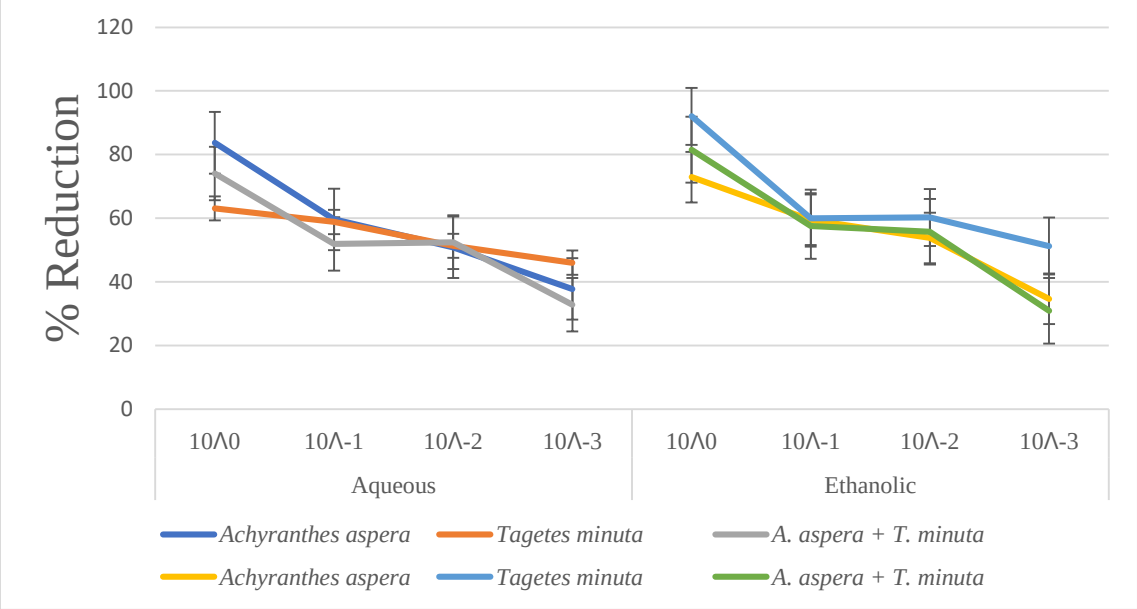

Figure 4.10: Percentage reduction in plumule lengths of Sorghum exposed to plant extract(s).

ANOVA determined that the main factors (concentration of the extract, solvent used, plant extract used and each plant species investigated) as well as some interactions had a statistically significant effect on plumule lengths of the test plants at $P \leq 0.05$ (Table 4.13, Appendix III). Since all but two P-values from the main factors and their interactions were less than 0.05 (Table 4.13, Appendix III), these factors have a statistically significant effect on plumule length of maize, millet, rice and sorghum.

Table 4.13: Effects of ethanolic and aqueous plant extracts on plumule lengths

Source of variation	F-Ratio	P-Value	Effect as at $p \leq 0.05$
Concentration	287.04	0.0000	Significant
Solvent used	12.46	0.0004	Significant
Plant extract used	13.32	0.0000	Significant
Plant investigated	222.16	0.0000	Significant
A: *Achyranthes aspera*	29.2	0.0000	Significant
T: *Tagetes minuta*	39.7	0.0000	Significant

A+T	46.8	0.0000	Significant

4.4.3 Effects of ethanolic and aqueous leaf extracts of *Achyranthes aspera* and *Tagetes minuta* plant extracts on radicle lengths

a) Maize

The highest percentage reduction in radicle lengths of maize was on aqueous extracts from *Tagetes minuta* (83%) in the original concentration. Ethanolic extracts from both *Achyranthes aspera* and *Tagetes minuta* and the aqueous extracts from a combination of these two plants had percentage reductions of 78% each. Aqueous extracts of *A. aspera* had a percentage reduction of 79% while ethanolic extracts from a combination of these two plants investigated resulted in a reduction of 80%. The other extract concentrations showed a similar pattern with reducing extract concentrations resulting in reduced activity was observed (Figure 4.11).

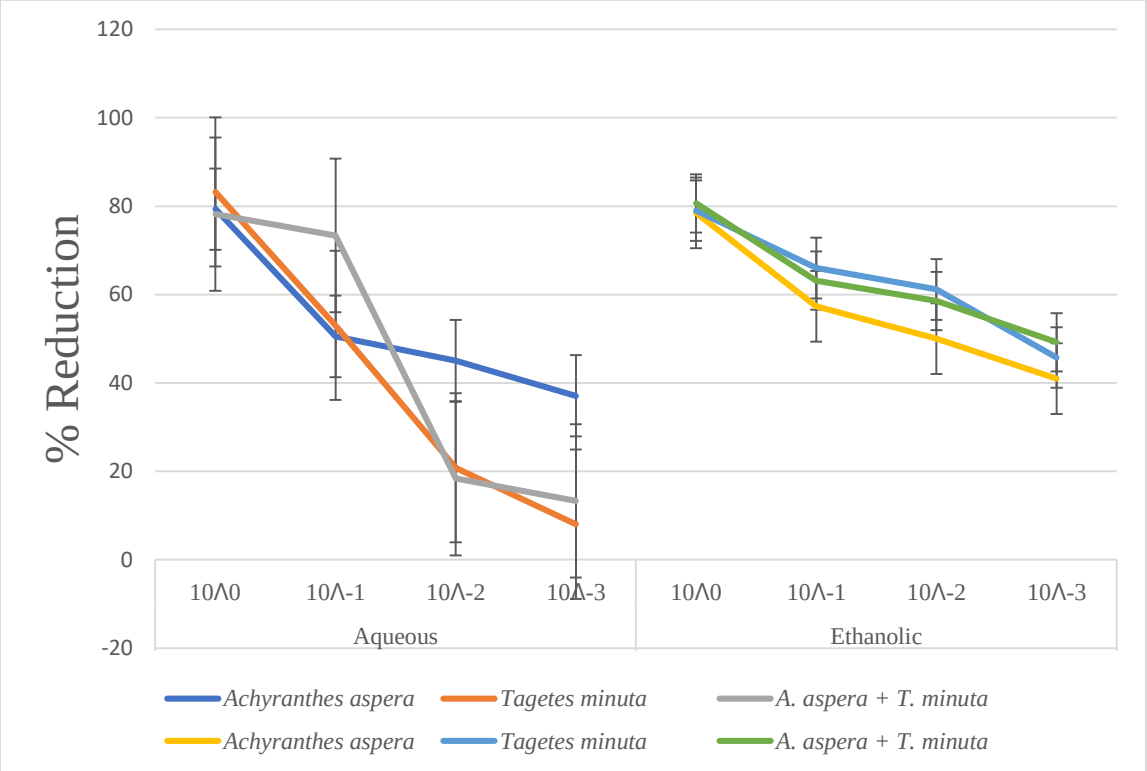

Figure 4.11: Percentage reductions in radicle lengths of Maize exposed to plant extract(s).

b) Millet

The radicle lengths of millet were more adversely affected by the extracts when compared to its plumules. This was evident from the percentage reduction of 94% at the highest and 79% at the lowest based on concentration 10^0. Those were obtained from aqueous extracts of *Achyranthes aspera* and ethanolic extracts of a combination of the two plants respectively. Reductions in radicle lengths of 82% and 91% respectively by the effect of *Tagetes minuta* aqueous and ethanolic extracts was noted in millet seeds (Figure 4.12). However, the effect of *Achyranthes aspera*'s ethanolic extracts as well as aqueous extracts obtained from a combination of the two plants was similar and could only reduce the lengths by 84% and 85% respectively. Generally, it was observed that the radicle lengths increased with a decrease in extract concentrations (Figure 4.12).

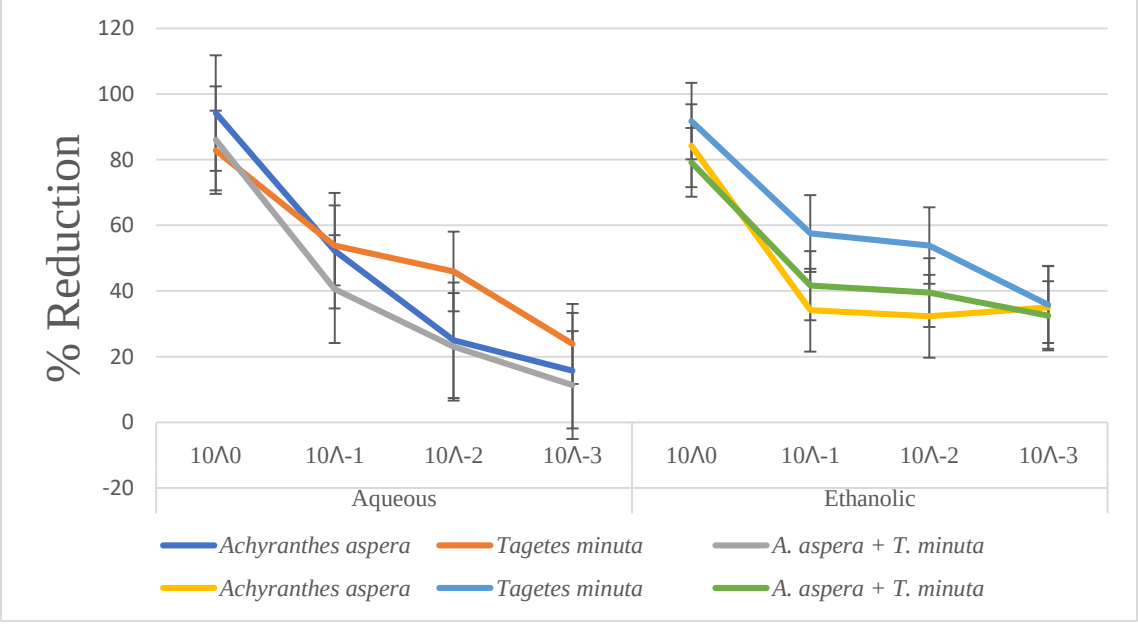

Figure 4.12: Percentage reductions in radicle lengths of Millet exposed to plant extract(s).

c) Rice

The highest percentage reduction in radicle lengths of rice was on aqueous extracts of *Tagetes minuta* (96%) at concentration 10^0 with the lowest percentage reduction being on both aqueous and ethanolic extracts from a combination of the two plants under investigation (77%). Aqueous and ethanolic extracts of *Achyranthes aspera* yielded percentage reductions of 80% and 82% respectively while ethanolic extracts of Tagetes *minuta* could only reduce the radicle lengths of rice by 79%. Results from the other extract concentrations followed a similar pattern. There was a reduction in the plant extract(s) effect on the radicle lengths as the concentration decreased (Figure 4.13).

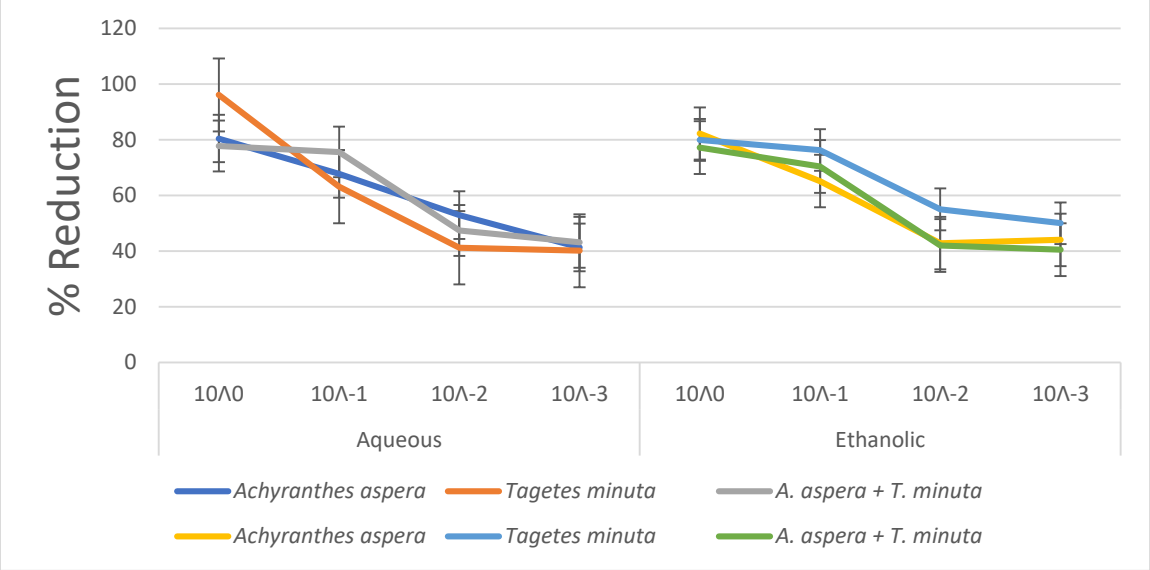

Figure 4.13: Percentage reductions in radicle lengths of Rice exposed to plant extract(s).

d) Sorghum

Based on the original concentration (10^0), the highest percentage reduction in radicle lengths of sorghum was obtained from ethanolic extracts of *Tagetes minuta* (97%) with the lowest being from both aqueous and ethanolic extracts of the combination of the two plants investigated (87%). Aqueous and ethanolic extracts of *Achyranthes aspera* yielded

percentage reductions of 96% and 87% respectively while aqueous extracts of *Tagetes minuta* could only reduce the radicle lengths of rice by 80%. In all the treatments, reducing activity with reducing extract concentrations, from 10^0 to 10^{-3} was observed (Figure 4.14).

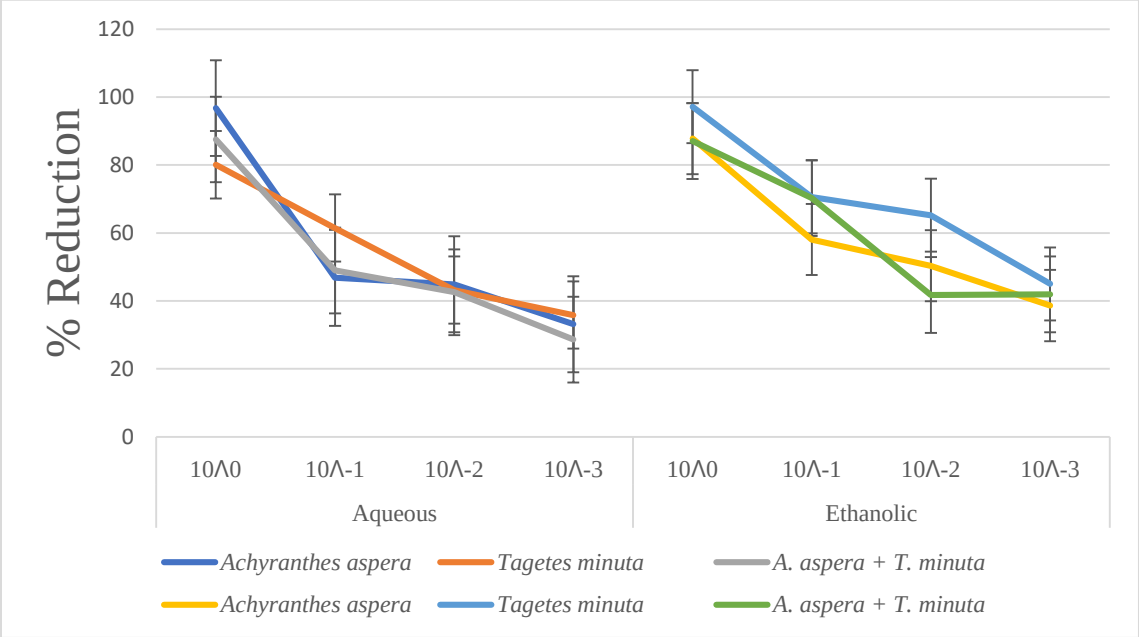

Figure 4.14: Percentage reductions in radicle lengths of Sorghum exposed to plant extract(s).

ANOVA was used to determine the effects of ethanolic and aqueous plant extracts on the radicle lengths of maize, millet, rice and sorghum. The p-values of the main factors ranged from 0.0000 to 0.0423 at 95.0% confidence level ($P \leq 0.05$) (Table 4.14, Appendix IV). Since all the P-values are less than 0.05, it means that these factors have a statistically significant effect on radicle length at 95.0% confidence level (Table 4.14, Appendix IV).

Table 4.14: Effects of ethanolic and aqueous plant extracts on radicle lengths

Source of variation	F-Ratio	P-Value	Effect as at $p \leq 0.05$
Concentration	287.04	0.0000	Significant
Solvent used	12.46	0.0000	Significant
Plant extract used	13.32	0.0423	Significant
Plant investigated	222.16	0.0000	Significant

A: *Achyranthes aspera*	29.2	0.0000	Significant
T: *Tagetes minuta*	39.7	0.0000	Significant
A+T	46.8	0.0000	Significant

The main factors (concentration of the extract, solvent used, plant extract used and the plant type under investigation) as well individual plant extracts (*Achyranthes aspera* and *Tagetes minuta*) and their interactions had a statistically significant effect on radicle length (Table 4.14, Appendix IV).

4.5 Phytochemical composition of *Achyranthes aspera* and *Tagetes minuta* leaf extracts

Qualitative phytochemical screening aimed to identify secondary metabolites present in *Achyranthes aspera* and *Tagetes minuta* leaf extracts. The phytochemicals that were screened included alkaloids, anthraquinones, coumarins, flavonoids, glycosides, phenols, quinones, saponins, steroids, tannins, and terpenoids.

All the eleven phytochemicals screened were present in at least one of the extracts. Alkaloids, coumarins, flavonoids, phenols, quinones, saponins, tannins and terpenoids were present in both plants (Table 4.15). Anthraquinones, glycosides and steroids were found only in *Achyranthes aspera*. Alkaloids, anthraquinones, coumarins, flavonoids, phenols, quinones, steroids, tannins and terpenoids were extracted by ethanol. Water extracted alkaloids, coumarins, flavonoids, glycosides, phenols, quinones, saponins, tannins and terpenoids as shown in Table 4.15.

Anthraquinones and steroids were absent in aqueous extracts while glycosides and saponins were also absent in ethanolic extracts of both plants. That means they were not

extracted by the solvents used during the extraction process. Table 4.15 below shows the phytochemical present (+) or absent (-) in the respective leaf extracts of *Achyranthes aspera* and *Tagetes minuta*.

Table 4.15: Phytochemicals in aqueous and ethanolic of *A. aspera* and *T. minuta* leaf extracts.

	Achyranthes aspera		***Tagetes minuta***	
Phytochemicals	Ethanol	Water	Ethanol	Water
Alkaloids	+	+	+	+
Anthraquinones	+	-	-	-
Coumarins	+	+	+	+
Flavonoids	+	+	+	-
Glycosides	-	+	-	-
Phenols	-	+	+	+
Quinones	+	+	+	-
Saponins	-	+	-	+
Steroids	+	-	-	-
Tannins	-	+	+	+
Terpenoids	-	+	+	+

Legend: + = Present, - = Absent

CHAPTER FIVE

DISCUSSIONS

5.1 Percentage yields of leaf extracts

After extraction, the mean percentage yield was higher for *Tagetes minuta* when compared to that of *Achyranthes aspera*. This can often be attributed to the leaf sizes of the two plants. *Tagetes minuta* has relatively smaller leaf sizes when compared to those of *Achyranthes aspera*. Smaller leaf sizes mean that the leaf content is concentrated due to the smaller leaf surface unlike larger leaf sizes. Therefore, upon extraction, the yield is likely to be higher. Aqueous extracts of *Achyranthes aspera* had a higher percentage yield with the lowest percentage yield being from ethanolic extracts obtained from the same plant. Extraction of *A. aspera* with water has been documented to yield higher when compared to using organic solvents (Tanveer *et al.,* 2019). It therefore validates what was achieved in this study. Ethanolic and Aqueous extracts of *Tagetes minuta* had nearly equal percentage yield. The differences in the percentage yields could have been brought about by the solvents used for extraction. Solvent used in extraction has been documented to be the single most important factor that influences the efficiency of extraction (Truong *et al.,* 2021).

5.2 Efficiency of solvents used for extraction

Water and ethanol were the solvents utilised for extraction in this study. Their efficiency varied as is evident from percentage yields obtained after extraction. The crude extracts obtained from water had a higher mean percentage yield when compared to that from ethanol. This can directly be linked to polarity of these two solvents used in this study. The polarity index of water is 10.2 which is higher than that of ethanol (4.3). This could explain why water had a higher mean percentage yield compared to ethanol. According to

Abubakar *et al.,* (2017) and Truong *et al.,* (2021), extraction efficiency favours solvents with highly polarity and is also evident from the results in this study. It has also been documented by Tanveer *et al.,* (2019) that extraction of allelochemicals with water is better than using organic solvents.

5.3 Antibacterial activities of crude extracts

Results from this study showed that aqueous and ethanolic leaf extracts of *Achyranthes aspera* and *Tagetes minuta* had antibacterial activity against *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 51299, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 all be it to varying degrees. The mean inhibition zones from *Escherichia coli* ATCC 25922 at 10^0 was 25.5 mm and 22.5 mm for *Achyranthes aspera* and *Tagetes minuta* respectively. The average zone of inhibition for *T. minuta* and *A. aspera* extracts on *Enterococcus faecalis* ATCC 51299 at 10^0 was 25.5 mm. Extracts of *A. aspera* and *T. minuta* had mean zones of inhibitions on *Klebsiella pneumoniae* ATCC 700603 of 15mm and 12.5mm respectively. The average zone of inhibition on *Pseudomonas aeruginosa* ATCC 27853 for *T. minuta* and *A. aspera* at 10^0 was 26 mm and 24 mm. The mean zone of inhibition for *S. aureus* ATCC 29213 at 10^0 was 38 mm and 40 mm for *A. aspera* and *T. minuta* respectively. Reducing extract effectiveness with reducing concentration was noted. The bioactivity of extracts in all the concentrations used (10^0 to 10^{-2}) on average was greater against *Staphylococcus aureus* ATCC 29213 followed by *Enterococcus faecalis* ATCC 51299 then *Escherichia coli* ATCC 25922 then *Pseudomonas aeruginosa* ATCC 27853 with little activity observed on *Klebsiella pneumoniae* ATCC 700603. This can be attributed to the structural, metabolic, and resistance-related differences among the different bacterial

species. Since gram negative bacteria have an outer membrane, it most likely aided in protecting these species from the effects of the plant extracts. That's why gram negative bacteria in this study appeared less affected than gram positive bacteria. It has also been documented that *K. pneumoniae* have enhanced efflux systems and enzymatic degradation capabilities of bioactive compounds in plant extracts (Paula-Ramos *et al.,* 2016).

Achyranthes aspera and *Tagetes minuta* are medicinal plants having been documented by earlier studies. They therefore possess antibacterial potential which has been proved as well in the current study. The crude extracts have been documented to confer higher efficacy as compared to pure or semi-crude plant extracts. This can be due to presence of several compounds within the extracts (Agrawal *et al.,* 2012). Rikisahedew *et al.* (2023) reported that the plant is rich in alkaloids, coumarins, phenolic compounds, flavonoids, terpenoids and thiophenes possess antibacterial activity as these compounds are responsible for its therapeutic properties.

The bioactivity of extracts in all the concentrations used (10^0 to 10^{-2}) on average was greater on *Staphylococcus aureus* ATCC 29213 followed by *Enterococcus faecalis* ATCC 51299 then *Escherichia coli* ATCC 25922 then *Pseudomonas aeruginosa* ATCC 27853 with little activity observed on *Klebsiella pneumoniae* ATCC 700603. Analysis for statistical significance on the effect of both aqueous and ethanolic leaf extracts of *A. aspera* and *T. minuta* had p-values which were less than 0.05 at $P \leq 0.05$. That means that the extracts of both plants had a significant effect on growth of the tested bacteria. Such observations concur with those of other authors (Kavishankar *et al.,* 2011; Tahir & Khan, 2012; Ali *et al.,* 2014, Panwar & Bhatt, 2014; Habtamu & Mekonnen, 2017; Opinde *et al.,*

2018; Jan *et al.,* 2019, Mishra *et al.,* 2020; Ganesh *et al.,* 2021; Abdoul-Latif *et al.,* 2022; Ahmad *et al.,* 2022; Abdel *et al.,* 2024).

According to Ahmad *et al.,* (2022), extracts of *Achyranthes aspera* can be utilised in combating antibiotic resistance brought on by bacteria that are resistant to many drugs. The author further reports that those extracts significantly inhibited *Staphylococcus aureus, Enterococcus faecalis, Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* The zones of inhibition were measured at 23 mm a similar observation to 25mm in the current study. *A. aspera* extracts have also been showed to have antibacterial activity on *Staphylococcus aureus* and *Pseudomonas aeruginosa* with inhibition zones of 16 mm and 14 mm respectively (Abdel *et al.,* 2024) relatively lower than the 38 mm and 24 mm recorded in this study. According to Habtamu & Mekonnen, (2017), *A. aspera* which is used as medicinal plant in different parts of Ethiopia showed antibacterial activities against *E. coli, P. aeruginosa, S. aureus* and *S. typhi* with inhibition zones of less or equal to 12 mm which is lower than what was recorded in this study. Similarly, extracts of *A. aspera* leaves from various solvents showed the antibacterial effect on *E. coli, E. aerogenes, S. aureus* and *P. aeruginosa* in the isolated from diabetes patients (Kavishankar *et al.,* 2011). Nargatti *et al.,* (2021) tested the extracts of *A. aspera* against 3 gram-negative bacteria (*E. coli, P. aeruginosa, K. pneumoniae)* and 2 gram-positive bacteria (*S. aureus* and *S. epidermidis*) and found out that they significantly inhibited their growth. *A. aspera* leaf extracts also exhibited antimicrobial activity against *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* which are involved in wound infections (Nigussie *et al.,* 2021). The difference in the results from these studies

and the current study can be linked to extraction solvents used as well as the different bacterial strains used by the different authors utilised.

Opinde *et al.,* (2018) reported that extracts from *T. minuta* showed antimicrobial activity against the testing microorganism; *E. coli, S. aureus* and *E. faecalis* with an inhibition zone ≥17.00 mm against all test microorganisms this was relatively similar to the ≥18.00 mm documented in this study. Panwar & Bhatt, (2014) as well as Tahir & Khan, (2012) also reported similar results. Ali *et al.,* (2014) investigated *T. minuta* leaf extracts and documented that they exerted remarkable activity against *Staphylococcus aureus* (MRSA) with the inhibition zones of 23 mm which was significantly higher than what was recorded in this study. Jan *et al.* (2019) also reported the antibacterial effect of *T. minuta* extracts *S. aureus* with an inhibition zone of more than 20 mm. This was also lower than what was recorded in the current study. Bacterial strains like *Escherichia coli, Staphylococcus aureus, Micrococcus leteus, Bacillus subtilis, Pseudomonas aeruginosa* and many more have been reported to be inhibited by extracts of *T. minuta* (Walia, & Kumar, 2020). Shahzadi & Shah, (2015), Santos *et al.,* (2017) and Abdoul-Latif *et al.,* (2022) also documented similar findings. The difference in the results from their studies and the current study could be due to extraction solvents used and the different strains of bacteria used.

This study utilized crude leaf extracts of *Achyranthes aspera* and *Tagetes minuta* which are known to possess both active and non-active substances. These include various phytochemicals which were found to be present in both their aqueous and ethanolic extracts. Plant parts such as leaves have been reported by various researchers to contain flavonoids and terpenoids that are scavengers for free radicals which in turn enhances the antibacterial activity of *Achyranthes aspera* and *Tagetes minuta* extracts (Cornelius &

Wycliffe, 2016; Rachuonyo *et al.,* 2016). According to Ahmad *et al.,* (2022), alkaloids, tannins, terpenoids and phenols which were also found in the extracts of *A. aspera* and *T. minuta* play an important role as resistance breakers aiding in combating antimicrobial resistance.

5.4 Allelopathic activities of crude extracts

Allelopathic activities of aqueous and ethanolic crude leaf extracts of *Achyranthes aspera* and *Tagetes minuta* was tested on seeds of selected maize, millet, rice and sorghum varieties. These are commonly grown field crops which are members of *Gramineae* family. Findings from this study showed that *Achyranthes aspera* and *Tagetes minuta* leaf extracts decreased germination percentages, radicle length and plumule lengths of the tested field crops. This is corroborated by the data of plumule and radicle lengths which were reduced significantly when compared with control. On average, the effects of the extracts was higher in rice followed by maize then sorghum while minimal on millet. This was noted from germination percentages whereby millet had the highest germination percentage of 89.44% while rice had the lowest percentage at 67.78%. Maize and sorghum had 87.50% and 85.83% respectively. These differences can be attributed to the thickness of the seed coat as well as presence and/or absence of detoxifying enzymes in the respective cereals. Rice is highly sensitive to allelochemicals because of its thin seed coat which allows for the rapid uptake of these chemicals. Maize has a relatively thicker seed coat compared to rice while millet and sorghum are naturally allelopathic as well having small seeds limiting the surface area of absorption hence more tolerant to allelochemicals.

The radicle lengths also appeared to be affected more compared to the plumule lengths. The reason for that could be early exposure, direct contact as well as the functionality of

the radicles. In contrast to plumules, which emerge later and are initially protected by seed tissues, radicles emerge early during germination and come into direct contact with the extracts, exposing them to lower quantities of extract. The primary function of radicles may also have worked to it detriment since their primary functions is water and nutrient uptake. The uptake of these extracts may have damaged some of their cells hence more affected. These findings were also made by Kemboi *et al.,* (2022) and can be attributed to the allelopathic effects from leaf extracts of *Achyranthes aspera* and *Tagetes minuta*. Arora *et al.*, 2015; Tanveer *et al.*, 2019; Safdar *et al.*, 2021; Sidhu *et al.*, 2023, among others have also documented similar information.

Ethanolic extracts had a relatively higher activity in this study when compared to aqueous extracts. This is despite Abubakar *et al.,* (2017), Tanveer *et al.,* (2019) and Truong *et al.,* (2021) documenting that extraction efficiency favours solvents having a higher polarity. In this case, water has a higher polarity when compared to ethanol. It is therefore possible that these observations may not be due to extraction capability of these two solvents but the possibility of ethanol toxicity since it was used when reconstituting the paste after solvent evaporation. Therefore, there is need to control the disposal of ethanolic substances in and around farm fields as has also been suggested by Koul *et al.*, (2022). Reducing activity with reducing extract concentration was also evident from results of this study which was also noted from earlier studies by Raoof & Siddiqui, (2012) and Kemboi *et al.,* (2022).

Earlier studies, have alleged that some extracts from weeds may possess some plant growth inhibitors as well as phytotoxic chemicals which are involved in allelopathy (Raoof & Siddiqui, 2012). They include growth regulators, some nutrients, phytochemicals and toxins (Mandal *et al.*, 2016; Alagesaboopathi, 2018; Kemboi *et al.*, 2022). They may also

contain various phytotoxic compounds that potentially work as herbicides to inhibit germination and growth of other plant species (Tanveer *et al.,* 2019). Their role in allelopathy has however not been well characterized.

Extract of *A. aspera* was demonstrated to be successful in inhibiting the emergence of pearl millet, guar and maize (Safdar *et al.,* 2021). Safdar *et al.,* (2015) also reported the effect of *Parthenium hysterophorus* L. in maize. In petri plates, *A. aspera* was found to inhibit germination percentage, root length and dried biomass of rice (Sidhu *et al.,* 2023). Sharma & Satsangi, (2012) also documented the allelopathic influence *Achyranthes aspera* (L.) on the germination and growth behaviour of *Oryza sativa* (L.). Tanveer *et al.,* (2014) reported a significant decrease in germination index of pearl millet, sorghum and maize in response to aqueous extracts of various plant parts of *A. aspera*.

T. minuta showed significant effects on germination percentages, radical length and plumule length of seedlings grown under lab conditions just as was the case in this study. It has also been known to develop a weedy habit and compete with several commercially significant crops, such as beans, maize and rice (Arora *et al.,* 2017). In petri plates, extracts of *Andrographis paniculata*, *Plectranthus ambonicus* and *Tagetes minuta* adversely affected the shoot and root length of rice, cowpea and green gram (Shakkira, 2022).

The effect of *A. aspera* and *T. minuta* among other weeds on major crops like wheat, sorghum, pearl millet, maize, rice among others has been documented by Arora *et al.,* (2015), Sadia *et al.,* (2015), Safdar *et al.,* (2015), Arora *et al.,* 2017, Patil & Sharma, (2021), Safdar *et al.,* (2021), Shakkira, (2022), Sidhu *et al.,* (2023), among others.

The results of this study have revealed the allelopathic properties of *Achyranthes aspera* and *Tagetes minuta*. This implies that the plant extracts under investigation can significantly inhibit the germination and possibly growth of these test plant species. It is clear from the current study that when *Achyranthes aspera* and *Tagetes minuta* are present in farm fields, there is a substantial probability that maize, millet, rice, and sorghum which are the four major staple food crops in Kenya will grow poorly. Therefore, there is need to control *Achyranthes aspera* and *Tagetes minuta* which grow as weeds to avert possible losses which often result from their allelochemicals. This could significantly lower yields, cause farmers to lose money, and ultimately lead to food insecurity.

5.5 Phytochemical composition of *Achyranthes aspera* and *Tagetes minuta* extracts

All the eleven phytochemicals screened were present in at least one of the extracts. Only *Achyranthes aspera* contained anthraquinones, glycosides, and steroids, indicating that *Tagetes minuta* does not contain these substances. But it's also possible that certain phytochemicals were not extracted or that some volatile compounds were lost when the extracts were concentrated to get rid of the extraction solvents. This study's results also indicated that the two solvents used for extraction were able to extract some phytochemicals differently. Alkaloids, anthraquinones, coumarins, flavonoids, phenols, quinones, steroids, tannins and terpenoids were extracted by Ethanol. Water on the other hand extracted alkaloids, coumarins, flavonoids, glycosides, phenols, quinones, saponins, tannins and terpenoids. Anthraquinones and steroids were absent in aqueous extracts while glycosides and saponins were also absent in ethanolic extracts from both plants. That means these compounds were not extracted by the respective solvents. The reason for that could be because these two solvents employed in the extraction procedure have different

polarities. It has been shown by some studies that the solubility of phytochemicals in each solvent can be impacted by the solvents' varying polarities (Abubakar *et al.,* 2017). It is also crucial to note that phytochemical(s) found in each plant bears a different chemical makeup, which then has a significant impact on how soluble the phytochemical(s) is in any particular solvent.

The phytochemical composition documented from the plants extracts from this study concurs with those of other authors before. Akram & Tembhre, (2016) reported that extracts of *Tagetes minuta* contained alkaloids, terpenoids, flavonoids, saponins, glycosides and tannins. Rikisahedew *et al.,* (2023) also reported presence of alkaloids, phenolic compounds, flavonoids, saponins, terpenoids and sterols in phytochemical analyses of *T. minuta* extracts. Extracts of *Tagetes minuta* showed the presence of flavonoids, saponins, alkaloids, and tannins (Opinde *et al.,* 2018). Similarly, Sadia *et al.,* (2015) reported that *T. minuta* contains a varying number of secondary metabolites that include flavonoids, terpenoids, saponins, thiophenes, monocyclic, bicyclic and acyclic monoterpenes. The preliminary phytochemical screening for secondary metabolites in *Achyranthes aspera* by Nigussie *et al.* (2021) indicated the presence of alkaloids, phenols, flavonoids, terpenoids, tannins and steroids in methanolic leaf extracts. Nargatti *et al.* (2021) also noted that the extracts of *A. aspera* contain mainly flavonoids, tannins, terpenoids, saponins, phytosterols and phenolic compounds. Similarly, Hayyat *et al.* (2020) documents that *A. aspera* is a reservoir of alkaloids, triterpenoid, diterpinoid, steroids, cardiac glycosides, and phenols. Abhang, (2024) found out that leaf extracts of *A. aspera* contain alkaloids, flavonoids, phenols, tannins, saponins and terpenoids which were also documented in the current study.

Phytochemicals of plants have been documented to have a wide variety of biological activities including antibacterial and allelopathic activities. The extracts' diversity of phytochemicals, such as flavonoids, tannins, saponins, and alkaloids, tempers the integrity of the cytoplasmic membrane function and the energy metabolism process by preventing the production of nucleic acids (Rachuonyo *et al.*, 2016). Sunday *et al.,* (2012) reported that phytochemicals responsible for allelopathy are also possessed by medicinal plants. This is also evident from the findings of this study, where aqueous and ethanolic leaf extracts of *A. aspera* and *T. minuta* showed antibacterial as well as allelopathic activities.

Previous research has indicated that alkaloids are toxic to foreign organism cells (Wintola & Afolayan, 2015). Tannins exert antibacterial effects primarily through two mechanisms: by forming hydrogen bonds with microbial enzymes, thereby inhibiting their activity, or by depriving bacteria of essential iron required for growth and metabolism (Ruddaraju *et al.,* 2020). Similarly, saponins have been reported to exhibit inhibitory effects on microbial cells, disrupting their structural integrity and function (Wintola & Afolayan, 2015).

Flavonoids, on the other hand, have been shown to interact with various molecular targets, including enzymes such as reverse transcriptase and DNA polymerase I, as well as membrane-associated proteins, resulting in membrane leakage and loss of cellular function. Moreover, flavonoids can interfere with the ATPase activity of plasma membranes, further impairing energy metabolism (Sunday *et al.*, 2012).

Extracts of *Tagetes minuta* containing flavonoids and other phytochemical constituents have been examined and shown to possess significant antibacterial activity against a wide range of microorganisms, including bacteria, fungi, and some nematodes (Opinde *et al.,*

2018). Hassanpour *et al.,* (2023) also documented that flavonoids possess antioxidant potential.

The current study findings document reduced germination percentages as well as plumule and radicle lengths of maize, millet, rice and sorghum in crude leaf extracts of *Achyranthes aspera* and *Tagetes minuta*. The presence of phytochemicals in these extracts can therefore be linked to these observations. Quinones have been reported to inhibit germination when present (Niaz *et al.,* 2020). According to Kordali *et al.,* (2022), presence of terpenoids in extracts causes reduction in germination percentage of seeds as well as seedling growth. Phenolic chemicals have also been shown to interfere with the actions of respiratory enzymes, preventing seeds from germinating (Kemboi *et al.,* 2022). Hayyat *et al.* (2020) in fact documents that weeds like *A. aspera* and *T. minuta* grow on abandoned farm fields and waste-lands creating problems to crops during germination and reducing efficiency of farm practices.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS.

6.1 Conclusions

Achyranthes aspera and *Tagetes minuta* grows as weeds in the farm fields of University of Eldoret but the present study has put to light evidence of their antibacterial activities as well as their allelopathic effects. This study reported mean percentage yield after extraction of *A. aspera* (21.16%) while *T. minuta* had 21.77%. Ethanolic extracts had a higher activity when compared to aqueous extracts. The bio-activity of extracts from both plants was highest on *Staphylococcus aureus* ATCC 29213 followed by *Enterococcus faecalis* ATCC 51299 then *Pseudomonas aeruginosa* ATCC 27853 then *Escherichia coli* ATCC 25922 with least effect on *Klebsiella pneumoniae* ATCC 700603.

Millet had the highest germination percentage of 89.44% while rice had the lowest at 67.78%. Maize and sorghum had 87.50% and 85.83% respectively. The extracts have also showed the effects of weeds on seed germination which is likely to impact growth and yields of crops in the farm field where they grow. The extracts were more detrimental on rice followed by Maize then Sorghum with minimal effect on Millet. The radicle lengths were also affected more when compared to the plumule lengths.

Achyranthes aspera and *Tagetes minuta* possess several phytochemicals such as alkaloids, anthraquinones, coumarins, flavonoids, glycosides, phenols, quinones, saponins, steroids, tannins and terpenoids which likely contribute to their antibacterial as well as allelopathic activities.

6.2 Recommendations

1. Since crude extracts were used in the study, further purification of extracts can be undertaken to produce semi-crude or pure plant extracts.
2. Aside from antibacterial and allelopathic activities, it is important for the antifungal activity, cytotoxicity and the mode of action of the extracts to also be determined.
3. The study only focused on allelopathic effects on seed germination. Seedling growth, crop productivity and yields can also be assessed.
4. There is also need for sensitization for the utilization of medicinal plants in the treatment of ailments caused by bacteria to aid in combating antibiotic resistance.

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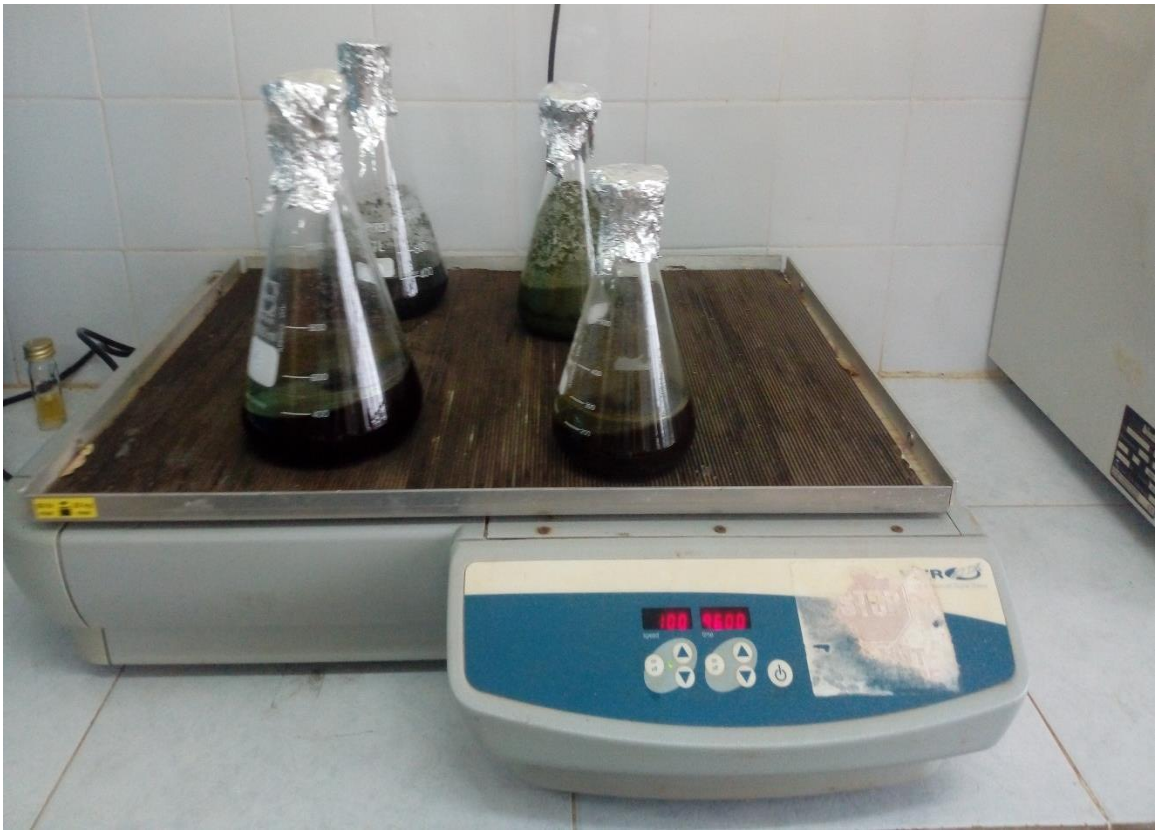
APPENDICES

Appendix I: Preparation and extraction of crude extracts

a. Collection of plants from the fields

b. Dried plant leaves

c. Ground plant leaves



d. Agitation of plant extracts in a shaker.

e. Filtration of plant extracts.

f. Concentration of extracts using a rotary evaporator

Appendix II: ANOVA Table for Inhibition Zone Diameters

Source of Variation	Sum of squares	Df	Mean Square	F-Ratio	P-Value
A: Concentration	4603.29	2	2301.64	296.49	0.0000
B: Solvent	11.2037	1	11.2037	1.44	0.2312
C: Plant species	18601.8	2	9300.9	1198.11	0.0000
D: Bacterial species	25669	4	6417.24	826.65	0.0000
INTERACTIONS					
AB	1.27407	2	0.637037	0.08	0.9213
AC	2146.58	4	536.644	69.13	0.0000
AD	962.6	8	120.325	15.5	0.0000
BC	37.3556	1	37.3556	4.46	0.0367
BD	325.481	4	81.3704	10.48	0.0000
CD	2121.31	8	265.164	34.16	0.0000
ABC	50.9926	4	12.7481	1.64	0.1656
ABD	128.319	8	16.0398	2.07	0.0413
ACD	403.311	16	25.2069	3.25	0.0001
BCD	173.385	8	21.6731	2.79	0.0062
ABCD	235.415	16	14.7134	1.9	0.0233
RESIDUAL	1397.33	180	7.76296		
TOTAL (CORRECTED)	56869.4	269			

Appendix III: ANOVA Table for Plumule Lengths

Source of Variation	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Concentration	564643	3	188214	287.04	0.0000
B:Solvent	8167.43	1	8167.43	12.46	0.0004
C:Extract	17471.7	2	8735.86	13.32	0.0000
D:Plant	437005	3	145668	222.16	0.0000
INTERACTIONS					
AB	5549.62	3	1849.87	2.82	0.0378
AC	13996.5	6	2332.75	3.56	0.0017
AD	62065.8	9	6896.2	10.52	0.0000
BC	12412.1	2	6206.07	9.46	0.0001
BD	92655.2	3	30885.1	47.1	0.0000
CD	10934.4	6	1822.39	2.78	0.0109
ABC	6631.47	6	1105.24	1.69	0.1211
ABD	71156.7	9	7906.31	12.06	0.0000
ACD	44626.7	18	2479.26	3.78	0.0000
BCD	2453.19	6	408.865	0.62	0.7116
ABCD	19402.7	18	1077.93	1.64	0.0435
RESIDUAL	735697	1122	655.701		
TOTAL (CORRECTED)	2.22E+06	1217			

Appendix IV: ANOVA Table for Radicle Lengths

Source of Variation	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:Concentration	534105	3	178035	372.64	0.0000
B:Solvent	44151.8	1	44151.8	92.41	0.0000
C:Extract	3031.53	2	1515.76	3.17	0.0423
D:Plant	1.25E+06	3	416576	871.92	0.0000
INTERACTIONS					
AB	31652.8	3	10550.9	22.08	0.0000
AC	21484.8	6	3580.8	7.49	0.0000
AD	116645	9	12960.6	27.13	0.0000
BC	13738.7	2	6869.33	14.38	0.0000
BD	49752.1	3	16584	34.71	0.0000
CD	10493.4	6	1748.89	3.66	0.0013
ABC	27629.3	6	4604.88	9.64	0.0000
ABD	69818.4	9	7757.6	16.24	0.0000
ACD	71623.4	18	3979.08	8.33	0.0000
BCD	13700.5	6	2283.42	4.78	0.0001
ABCD	85037.2	18	4724.29	9.89	0.0000
RESIDUAL	536057	1122	477.769		
TOTAL (CORRECTED)	3.11E+06	1217			

Appendix V: Book of Abstracts for ICRECC Conference

Allelopathic Potential of Achyranthes aspera and Tagetes minuta Leaf extracts on Seed Germination of Maize, Millet, Rice and Sorghum

Loyce Jeptoo · Pascaline Jeruto · Emilly Too · Lizzy Mwamburi

University of Eldoret (Kenya)

Weeds are a diverse group of non-economic plants that grow in places where they are not required or may grow in association with crops causing economic losses like reduced yields through competition or by releasing allelochemicals, in a process known as allelopathy. Achyranthes aspera and Tagetes minuta which grow as weeds in the fields, have been documented to possess allelopathic potential. Their impact increases with increase in the extract(s) concentrations. Losses due to weeds have been estimated to be even more than those caused by insect pests and diseases. The objective of this study was to determine the allelopathic potential of Achyranthes aspera and Tagetes minuta. Plant leaves were collected, shade dried followed by extraction using water and ethanol. The extracts were tested against maize, millet, rice and sorghum seeds. Five (5) surface sterilized seeds were placed in each Petri dish. 30 millilitres of test extracts were used with double distilled water set as positive control and DMSO set as negative control. Dilutions of extracts with double distilled water was prepared for concentrations of 10^0 to 10^3. To assess possible synergy, 15ml of each extract was mixed then dispensed to the petri dishes. They were laid out in a Completely Randomized Design (CRD) with three replications at $25 \pm 2°C$. The emerged plumule and radicle lengths were recorded after seven (7) days and germination percentages calculated. Millet had the highest germination percentage of 89.44% while rice had the lowest percentage at 67.78%. Maize and sorghum had 87.50% and 85.83% respectively. The ANOVA p-values of between 0.0000 and 0.0423 means that aqueous and ethanol extracts leaf extracts of Achyranthes aspera and Tagetes minuta had a statistically significant effect on plumule and radicle lengths of maize, millet, rice and sorghum at P âLd 0.05. It suggests that the extracts of Achyranthes aspera and Tagetes minuta interfered with the germination and growth of the test plants. This implies that there is a high likelihood for poor growth performance of these major staples in the presence of Achyranthes aspera and Tagetes minuta. The results of study may be used to assess allelopathic potential against other field crops as well as further studies on their potential effect on growth and development in the fields.

Keywords: Allelopathy · allelochemicals · Weeds · Achyranthes aspera · Tagetes minuta· ANOVA

Appendix VI: Publication

Allelopathic Potential of *Achyranthes aspera* and *Tagetes minuta* Leaf Extracts on Seed Germination of Maize, Millet, Rice, and Sorghum

Jeptoo Loyce, Pascaline Jeruto, Emily Jepkosgei Too, and Lizzy A. Mwamburi

Abstract

Achyranthes aspera is a weed of the family *Amaranthaceae* and grows to about 2 m high. *Tagetes minuta* is weed of the family *Asteraceae* that grows to a height of 1–2 m. The two plants are major weeds of cultivated crops. They have been documented to possess allelopathic activities. Studies have estimated that weeds generate greater losses compared to diseases and pests. That is why this research was carried out to determine the allelopathic potential of *Achyranthes aspera* and *Tagetes minuta* crude leaf extracts on the germination of maize, millet, rice, and sorghum seeds. Leaves were collected from farm fields and shade dried followed by aqueous and ethanolic extraction. Each petri dish contained five seeds that had been surface sterilized. Thirty (30) milliliters of extracts were utilized while distilled water was used as positive control. This was set up at 25 ± 2 °C in three replications adopting a completely randomized design. After 7 days, the lengths of the emerging plumule and radicles were measured, and the germination percentages were calculated. Significant inhibition of radicle and plumule lengths of maize, millet, rice, and sorghum ($p \leq 0.05$) was demonstrated by the extracts. Millet recorded the highest germination percentage (89.44%), while rice recorded the lowest at 67.78%. Generally, the extracts interfered with germination and

Authors Pascaline Jeruto, Emily Jepkosgei Too and Lizzy A. Mwamburi contributed equally to this work.

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N. Ronoh, C. Rono (eds.), *Rethinking Our Ecosystems*, Springer Proceedings in Geography and Sustainability, https://doi.org/10.1007/978-3-031-95781-9_7

Appendix VII: Similarity Report

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