ANTIBACTERIAL ACTIVITIES, ALLELOPATHIC EFFECTS AND PHYTOCHEMICAL COMPOSITION OF *Centella asiatica*, *Dichondra repens* AND *Hydrocotyle mannii*

BY

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A THESIS SUBMITTED TO THE FACULTY OF SCIENCE DEPARTMENT OF BIOLOGICAL SCIENCES IN PARTIAL FULFILLMENT FOR THE AWARD OF MASTER OF SCIENCE DEGREE IN MICROBIOLOGY

UNIVERSITY OF ELDORET, KENYA

2022

DECLARATION

Declaration by the Student

I declare that this thesis titled "ANTIBACTERIAL ACTIVITIES, ALLELOPATHIC EFFECTS AND PHYTOCHEMICAL COMPOSITION OF *Centella asiatica*, *Dichondra repens* AND *Hydrocotyle mannii*" is my own original work which I undertook and has never been submitted in any other institutions of higher learning. The literature in this work has been duly acknowledged in the text and a list of references provided. No part of this work has previously been presented for examination.

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DEDICATION

This work is dedicated to my late mother for her tender care and selfless nurture of my being.

ABSTRACT

Indigenous communities depend almost entirely on herbal remedies as the main source of their demands for primary healthcare. Medicinal plants like *Centella asiatica*, *Dichondra repens* and *Hydrocotyle mannii* have been used by residents of Nandi County for treating ear infections and abdominal ailments. More people died from antibiotic resistant bacteria than of HIV and AIDS in 2019. This is due to antibiotics being used in improper, irregular, and unreasonable ways. Approximately 60% of children are estimated to have experienced at least one episode of Otitis media (ear infections) by 7 years. In addition to having medicinal properties, *C. asiatica*, *D. repens* and *H. mannii* are weeds that may possess allelopathic properties. Crop losses caused by weeds are believed to be greater than those caused by insects, pests, and diseases. This study determined the antibacterial activities, allelopathic effects and phytochemical composition of *C. asiatica*, *D. repens* and *H mannii*. The plant leaves were collected, shade dried followed by extraction using water, diethyl ether, ethanol and ethyl acetate. Extracts were tested against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* to determine antibacterial activities as well on maize, oats, rice, sorghum and wheat seeds to establish their allelopathic effects. Susceptibility test was done by disc diffusion using six mm discs with desired extract concentrations (10^0 to 10^{-3}) with Ciprofloxacin as a positive control. Clear zones around the discs were measured as inhibition zone diameters. Allelopathic effects was tested by placing 10 surface sterilized seeds in each Petri dish. Twenty-four millilitres of the test extracts was used with distilled water as a positive control. The test extracts showed significant inhibitory effect against the test bacterial strains as well as test seeds which was evident from inhibition zone diameters and the plumule and radicle lengths. MANOVA determined that all the main factors and interactions with the exceptions of the interaction between plant type and bacteria species and the interaction between the concentrations, plant type and bacteria species used had a statistically significant effect on the inhibition zone diameters as well as plumule and radicle lengths at $P \leq 0.05$. Alkaloids, coumarins, flavonoids, glycosides, phenols, quinones, saponins, steroids, tannins and terpenoids were screened with only glycosides absent in any of the test plant extracts. This study concludes that the phytochemicals present in the extracts of the plants under investigation were responsible for the antibacterial and allelopathic activities of the plants to varying degrees. Water can be used for optimal yield of crude extracts. The plants had allelopathic effect on test plants and can be recommended as weed control agents. Medicinal plants have a promising future as most of them have not yet been investigated for their hidden potential which could be decisive in the development of newer and more effective antimicrobials as well as in controlling growth of other plant species.

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LIST OF ABBREVIATIONS

AOM – Acute Otitis Media

ATCC – American Type Culture Collections

CLSI – Clinical and Laboratory Standards Institute

CRD – Completely Randomized Design

CSOM – Chronic suppurative Otitis Media

DMSO - Dimethlsulphoxide

DOS – Doctrine of Signatures

KEMRI – Kenya Medical Research Institute

MDR – Multidrug Resistant

XDR – Extensive Drug Resistant

PDR – Pan-Drug Resistant

MANOVA – Multifactor Analysis of Variance

MIC – Minimum Inhibitory Concentration

NA – Nutrient Agar

OM – Otitis media

OME – Otitis Media with Effusion

PCV – Pneumococcal Conjugate Vaccines

RSV – Respiratory Syncytial Virus

SOM – Serous Otitis Media/Secretory Otitis Media

WHO – World Health Organization

ACKNOWLEGMENTS

My profound gratitude goes to my supervisors Dr. Pascaline Jeruto and Prof. Lizzy Mwamburi of University of Eldoret (UoE) as well as Dr. Richard Korir of Kenya medical research institute (KEMRI) for their efforts, contributions and support towards the completion of this research.

I would like to appreciate my father, brother, friends and relatives for their counsel, financial assistance, and continual prayers for me.

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

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Plants including weeds and their products possess medicinal and therapeutic activities which have been utilized in maintaining good health since the dawn of time (Jamshidi-Kia *et al*., 2018). Medicinal plants have a wide range of active phytochemicals which are of great interest in the past, current and future studies to obtain newer more effective antimicrobials (Singh, 2015). Medicinal plants are those plant species utilized in traditional or modern medicine that possess therapeutic ingredients in the treatment of human and/or animal illnesses (Ahn, 2017). Phytochemicals are active plant derived biochemicals and/or bio-compounds which are generated by most plants during primary and secondary metabolism which can impart dietary, biological and pharmaceutical advantages (Khalid *et al.,* 2018). Chemical substances which prevent microbial growth or kill the microorganisms are termed antimicrobial agents (Idris & Nadzir, 2017).

Currently, the use of traditional medicine is on the increase worldwide for many reasons among them being widespread antimicrobial resistance as well as undesirable side effects of some antibiotics (Ventola, 2015: *Li et al.,* 2017). Antimicrobial resistance is resistance over time to previously effective antimicrobials used to treat and/or control infectious microorganisms (O'Neill *et al.,* 2016; Murray *et al.,* 2022).

In addition to having medicinal properties, most plants have allelopathic properties and as such are treated as weeds. Weeds are a wide variety of non-economic plants which grow where they are not wanted or may grow alongside crops, resulting in economic losses such as decreased yields due to competition or the release of allelochemicals, a process known as allelopathy (Anwar *et al.,* 2019). Allelopathy is a biological phenomenon whereby organisms' releases allelochemicals which escapes into the environment and influence the survival, germination, reproduction as well as growth and development of neighbouring organisms (Bahadur *et al.,* 2015). Allelochemicals are phytochemical compounds liberated from some plants and impact other susceptible plants directly or indirectly (Nazir *et al.*, 2014). *Centella asiatica, Dichondra repens* and *Hydrocotyle mannii* grow as a weed community under shades and on moist soils forming almost one homogenous layer. The weed community is termed *Hydrocotylo-Centelletum asiaticae* association and is dominated by *C. asiatica* and *H. mannii* (Mosango, 2017).

1.2 Statement of problem

More people died as a result of antibiotic resistant bacteria than of HIV & AIDS in 2019 with sub-Saharan countries like Kenya worst affected (Murray *et al.*, 2022). Antibiotics have performed a key role in treating infections over a long period of time. However, inappropriate and irregular usage of antibiotics has caused the emergence of widespread antimicrobial resistance (Anand *et al.*, 2019). There are fewer active antimicrobials are now available to treat illnesses due to increased antibiotic resistance by multi-drug, extensive drug as well as pan-drug resistant microbes which are now virtually resistant to almost all the available antibiotics (Ventola, 2015). Some antibiotics also have undesirable side effects hence not commonly used (Chua *et al.*, 2015).

Antimicrobial resistance results in increase in morbidity hence long hospital stay leading to huge economic loses and mortalities (O'Neill *et al.*, 2016; Murray *et al.*, 2022). In Kenya, that economic loss due to this is estimated at an average of US$19.86 per-family, per-episode, both directly and indirectly (Tate *et al.*, 2009).

For instance, children between the ages of 6 and 24 months and 3-5 years are more likely to get ear infections due to exposure to environmental factors while attending day-care and/or Kindergarten (Qureishi *et al.*, 2014). By the time they are seven years, up to 60% of children have reportedly had at least one episode of otitis media (Todberg *et al.*, 2014). Otitis media, almost always attributed to ear infections has been associated with hearing impairment, Learning disabilities, attention impairments, and social adjustment issues and delayed speech development in children. The estimates of World Health Organization (2015) indicate that More than 5% of people worldwide suffer from minor hearing loss linked to otitis media (Argaw-Denboba *et al.*, 2016). Long-term use of antibiotics for treating it has been linked to the rise of otitis bacteria that is resistant to antibiotics which is of great public health concern worldwide (Saraca *et al.*, 2019).

Most plants with medicinal properties are weeds that affect agricultural production. It is therefore prudent that their growth is regulated in areas with economically important crops in order to avoid losses. About 24 percent of Kenya's gross domestic product is produced by the agricultural sector. It is also estimated that about 75% of Kenyans depend on agriculture either directly or indirectly (Ochilo *et al.*, 2019). According to estimates, losses from weeds exceed those from diseases and insect pests (Zohaib *et al.*, 2016). It is estimated that weeds can result in a decline of up to 35-80% in maize yields, 35-40% reduction in rice yield and 25-30% in the yield of wheat, depending upon types of weeds, weed density, management practices, duration of the competition as well as weather conditions (Zohaib *et al.*, 2016). The negative effects of weeds leads to increasingly lower yields, low income and eventually a decline in the economy making more farmers to shift and find alternative sources of income.

1.3 Justification

About 70-80% of the world's rural population use traditional medicines for general health care particularly in developing countries like Kenya, attributed to unavailability and/or inaccessibility of medicine, or due to undesirable side effects associated with some antibiotics (Chua *et al.,* 2015). Increased microbial resistance has been linked to emergence of widespread drug resistance as a result of irregular, inappropriate and irrational uses of antibiotics as well as the unavailability and/or inaccessibility of essential medicine (Anand *et al.*, 2019). In 2019, it is estimated that 5 million deaths were associated with antibacterial resistance with *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Streptococcus pneumoniae* attributed to over 3 million of those deaths (Murray *et al.,* 2022).

Plants including weeds have for long been depended by human beings to primarily meet medical needs in maintaining health and curing chronic and/or infectious diseases (Jamshidi-Kia *et al*., 2018). Plants with medicinal value are still effective today as they were thousands of years ago. In Kenya, many indigenous communities particularly those from the rural areas depend on medicinal plants almost entirely. Even when there is access to modern medicine, some still believe in the potency of herbal medicine while some opt to use a combination of herbal and conventional medicine (Yuan *et al.,* 2016). Their effectiveness is mainly attributed to their phytochemicals/secondary metabolites.

Unless measures are taken to control antimicrobial resistance by 2050, it is estimated to contribute to over 10 million deaths annually costing US$100 trillion with low and middle-income nations like Kenya more negatively affected. (O'Neill *et al.,* 2016). As per the estimates of Global Burden of Disease Study (GBD, 2015), 3,200 people died in

2015 as a result of otitis media (Argaw-Denboba *et al.,* 2016). Because a single plant has a vast range of phytochemicals, resistance to such botanicals is believed not to be as widespread as in antibiotics which have a few active ingredients (Ruddaraju *et al.,* 2020).

Doctrine of Signatures theory suggests human discovery of medicinal plants. It states that, just as form recapitulates function, physical characteristics of certain plants can reveal their therapeutic value (Bennett *et al.,* 2007). This can explain the use of *C. asiatica, D. repens* and *H. mannii* by some residents of Nandi County for medicinal purposes like treating abdominal pains (Jeruto *et al.*, 2015), ear infections (indigenous knowledge) and as an antivenin (Owuor & Kisangau, 2006).

Weeds alter the normal growth and induce a decrease in crop output by interfering with certain metabolic procedures stemming from weed-crop competition and allelopathy (Anwar *et al.*, 2019). Most studies have undermined decrease in crop output due to weed-crop allelopathic interactions (Ali *et al.*, 2015). There is a shortage of information highlighting the negative effects of weeds on crops due to weed-crop allelopathic interactions even though estimates indicate that losses from weeds exceed those caused by insect pests and diseases (Zohaib *et al.*, 2016). Weeds may cause reduction in field crops yields, depending on management practices, the duration of the competition, the weed types, the density of the weeds and the weather conditions (Zohaib *et al.,* 2016).

This study therefore sets to inform and document the role of *Hydrocotylo-Centelletum asiaticae* weed community as a new source of antimicrobial products in the management of otitis media and highlight their allelopathic effects on other crops which could be an alternative method of weed management to the environmentally hazardous herbicides.

1.4 Objectives

1.4.1 Broad objective

To determine the antibacterial activities, allelopathic effects and phytochemical composition of *Centella asiatica, Dichondra repens* and *Hydrocotyle mannii*.

1.4.2 Specific objectives

a) To determine antibacterial activities of *C. asiatica*, *D. repens* and *H. mannii* leaf extracts against *Escherichia coli* ATCC 25922, clinical isolate of *Pseudomonas aeruginosa* and *Staphylococcus aureus* ATCC 25923.

b) To determine the allelopathic effects of *C. asiatica, D. repens* and *H. mannii* leaf extracts on maize, oats, rice, sorghum and wheat.

c) To screen for the qualitative presence of phytochemicals in *C. asiatica*, *D. repens* and *H. mannii* leaf extracts used against test plants and bacteria.

1.5 Hypothesis

a) Leaf extracts of *C. asiatica, D. repens* and *H. mannii* are not active against *Escherichia coli* ATCC 25922, clinical isolate of *Pseudomonas aeruginosa* and *Staphylococcus aureus* ATCC 25923.

b) *C. asiatica, D. repens* and *H. mannii* are not allelopathic to maize, oats, rice, sorghum and wheat.

c) The phytochemicals present in *C. asiatica, D. repens* and *H. mannii* are not potent against test plants and bacteria.

CHAPTER TWO

LITERATURE REVIEW

2.1 Medicinal plants

A medicinal plant is one that is administered for a specific condition, used with the intention of maintaining health, or both, whether in traditional medicine or in modern medicine at a regional or local scope (Ahn, 2017). Over the past two decades, the use of traditional medicine has increased tremendously with the World Health Organization (WHO, 2015) encouraging the use of medicinal plants, because they have been proven to be easily accessible, safe, less toxic, efficient and reliable (Kalaimagal & Umamaheswari, 2015). According to the WHO, over 80% of the world's population today rely on traditional or herbal medicine as the main source of their demands for primary healthcare (Chua *et al.*, 2015).

Traditional medicinal plants contain substances that can be used in both industrialized and developing nations to treat chronic and infectious ailments (Kalaimagal & Umamaheswari, 2015). They also possess specific characteristics like synergistic effects, in which plant elements may combine with one another and the resulting molecules may produce greater result (Jamshidi-Kia *et al*., 2018). Antibiotics used in combination with plant extracts produce a synergistic effect which can be employed to manage microbial drug resistance.

Traditional herbal medicines are mostly prepared from the leaves, roots, bark, seed, fruit, flowers or even the whole plant in the case of herbs and lower plants (Jamshidi-Kia *et al*., 2018). They are commonly administered orally, inhaled or directly applied to the affected area (Jeruto *et al.,* 2015). Medicinal plants like *C. asiatica L.* Urban, *D. repens* and *H.*

mannii Hook f. therefore offers an alternative solution for treating human diseases (Singh, 2015; Pandey *et al*., 2020). They also form the foundation of commercial drugs used in contemporary medication today (Zang *et al*., 2013).

2.1.1 *Centella asiatica*

Centella asiatica L. Urban is a small, edible, prostrate, faintly aromatic, brown and greenish-yellow leafy ubiquitous herbaceous plant of the *Apiaceae* family (Plate 2.1). *C. asiatica* has many common names including Indian Pennywort, Marsh Pennywort or Gotu kola (Singh, 2015), Mungutab beliot ne sing'ortot (Jeruto *et al.*, 2015) among others. There are about 50 species in the Genus *Centella*, most of which grow in tropical and subtropical climates of Asia, Oceania, Africa and America (Kunjumon *et al.*, 2022). They thrive between 0 and 2500 meters above sea level in damp environments and shaded areas (Singh, 2015).

Plate 2.1: *Centella asiatica* L. Urban leaves (Kemboi, 2018).

Centella asiatica is creeping perennial herb having long, slender and tender horizontal reddish stolons, rooted at nodes with long nodes and internodes. The leafstalk is very slender and grows between 2 and 6 inches high, beginning at the base and branching out to form individual leaf clusters that resemble roses (Singh, 2015). The 1-3 long petioled leaves (2-6cm long and 1.5-5cm wide) arise from each node of the stems. They are green, crowded, cordate, orbicular or round reniform, 1.4 cm by 1.7 cm with crenate or dentate margin, sheathing leaf base and globous on both sides (Singh, 2015). The plant flowers between August and September are sessile in simple umbels with 3-4 white or light purple-to-pink petals bearing about 0.4 cm long oval to globular shaped fruit (Zahara *et al.,* 2021). *Centella asiatica* can attain a height of up to 6 inches and creates a dense green carpet by flourishing in shaded, marshy, damp, and wet regions like paddy fields and river banks (Singh, 2015). Depending on the environmental conditions, *C. asiatica* plants can have very different shapes.

Centella asiatica is found growing in ruderal areas and abandoned farming regions, especially those with moist soils and shades. *Centella asiatica* is closely associated with *Dichondra repens* and *Hydrocotyle mannii* to form *Hydrocotylo-Centelletum asiaticae* association (Mosango, 2017).

Centella asiatica plant is a popular herb that is utilized in different forms in most tropical and subtropical countries with its leaves being the most used part for medicinal purposes (Kunjumon *et al.,* 2022). However, the International Union for Conservation of Nature and Natural Resources (IUCN) has categorized it as an endangered species due to overexploitation prompted by its medicinal potential (Singh, 2015).

2.1.2 *Dichondra repens*

Dichondra repens J. R. Forst. & G. Forst. is a small, prostrate, low growing, creeping perennial plant with persistent leaves that spreads by underground runners and grows independently or alongside turfgrass in subtropical and Mediterranean climates (Song, Wang, Zhang & Xuan, 2015; Mosango, 2017). Additionally, *Dichondra repens* may be found growing in somewhat shaded regions and is closely related to *Centella asiatica* and *Hydrocotyle mannii* forming *Hydrocotylo-Centelletum asiaticae* association, a weed community (Mosango, 2017).

The leaves are small, dark green in colour and kidney-shaped to circular, 5 to 25 mm long and slightly wider (Plate 2.2). The leaf has a rounded apex, a heart-shaped (cordate) base and a stem of up to 50 mm long. The tiny, yellow-green inconspicuous flowers occur between September and February and are followed by a two-lobed capsule (Dawson, 2014). Due to its creeping habit, the overall appearance is that of a flat cover with a dense neat velvety appearance which spreads readily on the ground throughout the year (Song *et al.*, 2015).

Plate 2.2: *Dichondra repens* plant (Kemboi, 2018).

Johann Reinhold Forster and Georg Forster, two German naturalists, originally collected and recorded the described *Dichondra repens* in New Zealand into *Convolvulaceae*, the Morning Glory Family (Song *et al.*, 2015). In Australia and New Zealand, it is referred to as Kidney Weed and Mercury Bay Weed, respectively (Dawson, 2014), Dichondra Pony Foot, Lawn Leaf (Cardin, Delecolle, & Moury, 2005) as well as Nalulanda in western Kenya (Luyha) (Odhiambo *et al.,* 2011).

Dichondra repens is highly resistant to diseases, can grow in warm-rainy to arid climate and can adapt in shadow to full sun environments. This stoloniferous herb can be found in forests, woodland, grasslands or in lawns where it may be planted or can grow as a weed (Dawson, 2014). It is the most dominant species in the grassland habitat of Nabkoi Forest in Kenya alongside two grasses *Digitaria scalarum* and *Pennisetum cladestinum* (Wanjohi *et al.,* 2017).

2.1.3 *Hydrocotyle mannii*

The genus *Hydrocotyle* is made up of about 100 plant species growing in tropical and temperate regions worldwide. *Hydrocotyle mannii* Hook f. is a creeping perennial and semi-aquatic plant species that reproduce by sending roots from stem nodes. Especially in the Guineo-Congolian and Sudano-Zambesian phytogeographical areas, *H. mannii* are widely distributed throughout tropical Africa (Mosango, 2017).

Hydrocotyle mannii has simple leaves with a modest base leafy outgrowth which are round to kidney shaped. It is commonly called Water pennyworts, Indian pennywort or Mungutab beliot ne chabai in Nandi dialect (Jeruto *et al.,* 2015).

Its flower clusters are simple and flat-topped or rounded (Plate 2.3). They have long creeping stems that often thrives under shades and on moist, moderately organically rich soil. forming dense mats near ponds, rivers, marshes or on roadsides (Mosango, 2017).

Plate 2.3: *Hydrocotyle mannii* leaves (Kemboi, 2018).

Hydrocotyle mannii is closely associated with *C. asiatica* and *D. repens* forming a weed community termed the *Hydrocotylo-Centelletum asiaticae* association (Mosango, 2017).

2.2 Uses of *Centella asiatica*, *Dichondra repens* and *Hydrocotyle mannii*

Traditional medicine has treated viral and chronic ailments with medicinal plants (Kalaimagal & Umamaheswari, 2015). *Centella asiatica* is among the valuable plants with a variety of traditional, medicinal and therapeutic values. It is utilized almost all over the worldwide as a source of medicine, food and beverages where it is consumed in different forms for example as juice, tea, pills or capsules (Mahomoodally, 2013).

Over time, the usage of *C. asiatica* in foods and drinks has increased due to its positive benefits on health, including its antibacterial, anti-inflammatory, antioxidant, and wound-healing properties (Restuati & Diningrat, 2018). In India, this herb is beneficial in improving memory, treating mental fatigue, skin ulcers, rash, anxiety, abdominal tumour, cataract, eye problems, fever, cholera and eczema (Pandey *et al*., 2020). Its leaves have been used as a herbal remedy to alleviate abdominal aches. 100g of freshly collected leaves are ground into a paste, and 20g are then applied topically to the troublesome area three (3) times per day till recovery (Jeruto *et al.*, 2015). *Centella asiatica* has been known since ancient times that it can treat syphilis, tuberculosis mental fatigue, anxiety, wounds and leprosy (Nazir *et al.,* 2014). It can be eaten raw, where it can also be cooked and served with sweet potatoes and coconut milk to lessen the bitterness of the plant (Chandrika *et al.,* 2015).

Centella asiatica, is gaining a lot of interest for its potential use in medicine, including its ability to treat wounds, act as an antioxidant, improves memory as well as having cytotoxic effect on liver tumour cells (Hussin *et al.*, 2014). According to Ren *et al.*, 2016, treating ovarian cancer cells with 40 ug/ml concentration of asiatic acid reduced their viability by up to 50%. It also showed cell cycle arrest and increased apoptosis by 7-10 folds. The plant is used to treat bronchitis asthma, bronchitis, respiratory problems, tuberculosis, swollen throat, tonsillitis and nose bleeding (Pandey *et al*., 2020). *Centella asiatica* is also documented to be effective in treatment of stomach ulcers, epilepsy, cholera, jaundice, diarrhoea, hepatitis, measles, smallpox, asthma, urethritis, syphilis, renal stones, rheumatism, leprosy and other skin diseases (Zahara *et al.*, 2021). It is used to cure a variety of conditions in Bangladesh, including dog bites, asthma, itching, leucorrhoea, malaria, and wounds (Sushen *et al.*, 2017). In Nepal, it is used for treating Childhood tidal fevers, measles, eye problems, swollen joints, rib pain, abdominal pain and cancer (Pandey *et al.*, 2020). Vitamins A (retinol), B1 (thiamine), B2 (riboflavin), B5 (niacin), C (ascorbic acid), and carotene are all abundant in *C. asiatica*. It is further associated also high in amino acids such as serine, alanine, threonine, aspartic, histidine, and lysine (Zahara *et al.*, 2021).

The plant leaves of *D. repens* have been utilized by the Luo community as an antivenin by rubbing onto snakebites as an antidote to "remove snake fangs" (Owuor & Kisangau, 2006). Additionally, it is employed as a potted plant for house decoration (Cardin *et al.*, 2005). *Dichondra repens* has been used in treating skin wounds and ringworms where fresh leaves are pounded fresh with oil or Ghee and the paste applied externally to the site (Odhiambo *et al.*, 2011). It has also been used to cure jaundice and dysentery in China (Song *et al.*, 2015).

Leaves of *H. mannii* have been used as herbs to treat abdominal pains by soaking 100 g of freshly picked leaves in one litre of water. 250ml of the infusion is then taken daily three times until full recovery (Jeruto *et al.*, 2015).

2.3 Phytochemical composition

Phytochemicals are secondary metabolites which occur naturally and are synthesized by plants during their normal growth and development. Phytochemicals are biological compounds produced by plants such as medicinal plants, vegetables and fruits, throughout primary and secondary metabolism and play important role in their defence system like protection against diseases. Numerous phytochemicals have proven dietary, biological, and medicinal properties (Khalid *et al.*, 2018). The most fundamental phytoactive biochemicals are alkaloids, flavonoids, tannins, coumarins, terpenoids, saponins, phenols, quinones, among others (Njeru *et al.*, 2013).

2.3.1 Alkaloids

Alkaloids are naturally occurring organic substances that contain the building blocks of nitrogen, carbon, hydrogen, and occasionally oxygen, sulphur, and other elements like chlorine, bromine, and phosphorus (Girdhar *et al.,* 2015). They are bitter in taste, colourless, crystalline or liquid at room temperature, toxic in nature and are widespread in many medicinal plants. Alkaloids can naturally be produced by many organisms including bacteria, fungi, plants, and animals. An alkaloid hydrocotylin ($C_{22}H_{33}NO_8$) has been isolated from *Centella asiatica* (Pal & Pal, 2016).

Presence of a basic nitrogen atom is the only unifying feature which can be used to classify alkaloids into three categories namely, True-alkaloids e.g. atropine and nicotine, Proto-alkaloids e.g. adrenaline, ephedrine, as well as Pseudo-alkaloids e.g. caffeine and theobromine (Roy, 2018). True alkaloids have a heterocyclic nitrogen-based ring and are derived from amino acids. Proto-alkaloids have nitrogen atoms obtained from an amino acid that is not a component of the heterocyclic ring while Pseudo-alkaloids do not originate from amino acids (Fielding *et al.*, 2020).

Alkaloids are produced by approximately 25% of angiosperms and gymnosperms with over 8000 natural compounds currently classified as alkaloids (Fielding *et al.*, 2020). The majority of alkaloids exhibit a variety of pharmacological effects like antimalarial (e.g. quinine), antibacterial (e.g. chelerythrine) (Cushnie *et al.*, 2014), antifungal (Khan *et al.*, 2017), antiasthma, anticancer (e.g. homoharringtonine) (Kittakoop *et al.,* 2014), antihyperglycemic activities (e.g. piperine), analgesic (e.g. codeine), central nervous stimulant (e.g. brucine), central nervous depressant (e.g. morphine) (Brook *et al.,* 2017), antihypotensive (e.g. ephedrine) antihypertensive (e.g. reserpine) (Cushnie *et al.,* 2014) and antiviral activity (Fielding *et al.,* 2020).

Alkaloids for example isopteropodine and pteropopine have antimicrobial activity promoting white blood cells to dispose pathogenic microorganisms (Njeru *et al.*, 2013). Caffeine showed substantial activity against *Pseudomonas aeruginosa* by reducing the secretion of its virulence factors as well as significantly inhibiting its biofilm formation (Chakraborty *et al.*, 2020). Alkaloids having antiviral activity include berberine (chikungunya and hepatitis C), palmatine (zika), tomatidine (dengue), michellamine (HIV) and oxymatrine (influenza A) (Fielding *et al.*, 2020).

Coronaviruses in both humans and animals have been reported to be broadly inhibited by alkaloids. They demonstrated an affinity to bind to the SARS-CoV-2 spike protein's receptor-binding domain, preventing it from attaching to the host cell. Alkaloids are intriguing substances that may be used as bioactive agents against SARS-CoV-2, as evidenced by this (Fielding *et al.*, 2020).

Alkaloids are now used in both conventional and modern medicine as well as building blocks for pharmaceuticals. In traditional medicine, quinine-rich cinchona bark has been used for treating malaria and in modern medicine, homoharringtonine and vinblastine has been exploited for cancer treatment (Cushnie *et al.*, 2014). Although they have a marked therapeutic effect in small quantities, alkaloids like atropine and tubocurarine can also be toxic to humans (Roy, 2013). Other alkaloids, such as cocaine, caffeine, and nicotine, have stimulant properties and have been used as both illicit and recreational substances (Cushnie *et al.*, 2014).

2.3.2 Coumarins

Coumarins are polyphenolic colourless and crystalline oxygenated heterocyclic compounds (Figure 2.1) which was first isolated from *Dipteryx odorata Willd* (coumaroun). As a heteroside or free forms, coumarin and its derivatives are naturally occurring chemicals that are found in large quantities in plants obtained from about 600 genera (Kupeli Akkol *et al.*, 2020). According to their chemical diversity and complexity, coumarins can be divided into simple coumarins (e.g. osthole), biscoumarins (e.g. dicoumarol), isocoumarins (e.g. thunberrginols), furanocoumarins (e.g. psoralen), pyranocoumarins (e.g. grandivittin) and phenylcoumarins (e.g. isodispar B) (Rohini & Srikumar, 2014).

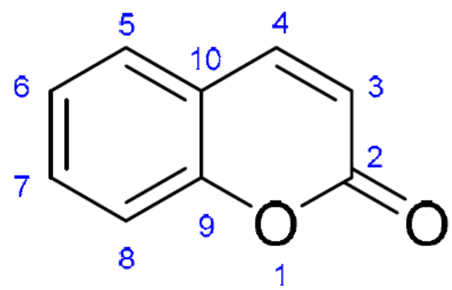

Figure 2.1: Structure of coumarin nucleus (Rohini & Srikumar, 2014).

Biological and pharmacological activities of coumarin and its derivatives including anticoagulant (e.g. dicoumarol), antibacterial (e.g. antoghenol, grandivittin), antifungal (e.g. psoralen), anti-inflammatory (e.g. isodispar B, columbianedin), neuroprotective (e.g. osthole), antidiabetic (e.g. thunberrginols), antiviral (e.g. inophyllum A, B, C, E, G_1, G_2, P), anticancer (e.g. osthole) as well as cardiovascular and hepatoprotective (e.g. osthole) (Annunziata *et al.*, 2020). Chronic illnesses like mononucleosis, toxoplasmosis, Q-fever, mycoplasmosis, and chronic brucellosis have all been managed with coumarins. By attaching to the plasma proteins and activating macrophages and proteolysis, they have strong edema protective functions and can be utilized to treat lymphedema, elephantiasis, and other high protein edema disorders (Rohini & Srikumar, 2014).

2.3.3 Flavonoids

Flavonoids are benzo-γ-pyrone derivatives with different numbers of hydroxyl substitutions in their structures (Chua *et al.*, 2015). They primarily contain glycosides, which are conjugated aromatic systems bonded to sugar (Njeru *et al.*, 2013). These bioactive substances are widely present in foods with a plant origin (Kozłowska & Szostak-Wegierek, 2014). Their regular consumption is associated with reduced risk to microbial infections, allergies, cancer, cardiovascular diseases, neurodegenerative disorders, diabetes, inflammations, reducing capillary fragility and lowering arterial pressure (Njeru *et al.*, 2013; Weston & Mathesius, 2013; Ullah *et al.*, 2020).

According to their degree of unsaturation, chemical composition, and level of carbon ring oxidation, flavonoids are categorized as anthocyanidins, isoflavonoids, flavanones, flavanonols, flavans, chalchones, and flavanones (Figure 2.2) (Ullah *et al.*, 2020).

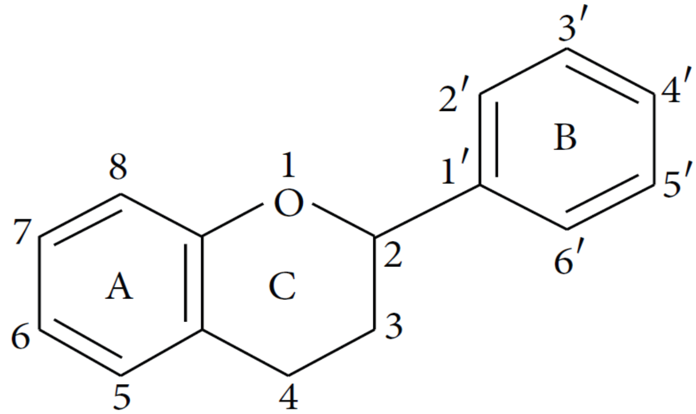

Figure 2.2: Basic flavonoid structure (Ullah *et al.*, 2020).

Three hydroxyl groups are present in the majority of naturally occurring flavonoids, two of which are located on ring A at positions five and seven and one of which is located on ring B at position three (Ullah *et al.,* 2020). In response to infection, plants produce flavonoids, which have potent antimicrobial properties with those lacking hydroxyl groups (-OH) on their structure said to be more active than those having -OH (Mishra *et al.,* 2013). Flavonoids such as 3-β glucosyl quercetin, kaempferol, 3-β glucosyl kaempferol, 7- β- glucosyl kaempferol & quercetin have been isolated from the leaves of *Centella asiatica* (Singh, 2015). Quercetin has anti-oxidant properties that stop macrophages from oxidizing low-density lipoproteins and metal ions (Duthie & Morrice, 2012; Njeru *et al.,* 2013). They control various important enzymatic pathways and functions, as well as gene expression. They have been documented to possess antioxidant, antiviral and antibacterial properties. Additionally, flavonoids influence how stable, sensorial, and healthy foods become. (Santos-Buelga & Feliciano, 2017).

Flavonoids apigenin and luteolin have been documented to have antibacterial activity against clinical strains of *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Alibi *et al.,* 2021).

The roots of specific plants emit flavonoids into the rhizosphere, where they participate in allelopathic interactions between plants. When compared to other cultivars in both lab and greenhouse experiments, the Japanese cultivar lucerne was found to be extremely inhibitive of seedling germination and growth linked to large flavonoid concentrations. However, their role in allelopathic interference has not been well characterized (Weston & Mathesius, 2013).

2.3.4 Phenols

One or more hydroxyl groups are joined to an aromatic hydrocarbon group to form phenols, a type of aromatic chemical molecules (Figure 2.3). It has the chemical formula C_6H_5OH and a hydroxyl group (-OH) attached to a phenyl ring as its main structural component (Knob & Pilato, 2013).

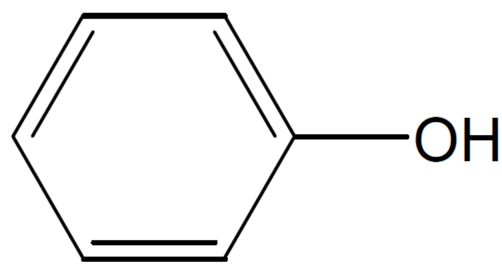

Figure 2.3: Structural formula of a phenol (Knob & Pilato, 2013).

The primary components of plant meals are phenolics, which can be found in fruits, vegetables, grains, legumes, as well as beverages like tea, coffee, beer, and wine (Shahidi & Ambigaipalan, 2015). Plant phenols are involved in defense against pathogens and parasites. Some phenols also such as Xylenol kills microorganisms by inactivating enzymes, disrupting of cell membranes as well as precipitating proteins. This properties can be utilised for making antiseptic disinfectants (Franklin & Snow, 2013).

Some phenolic acids have antibacterial activities. Among numerous phenolic compounds, garllic acid demonstrated the strongest antibacterial activity against a variety of bacteria among them are *S. aureus*, *Pseudomonas aeruginosa*, *Moraxella catarrhalis*, *Escherichia coli*, *Streptococcus pneumonia* and *E. faecalis*. Compared to ampicillin, which had minimum inhibitory concentrations of 3.2 and 0.1 g/ml for *Escherichia coli* and *S. aureus*, caffeine had a minimum inhibitory concentration of 1600 g/ml (Aldulaimi, 2017).

Manilkara hexandra (Roxb.) Dubard methanolic leaves' extract, which is rich in phenolic compounds, has a significant inhibitory impact on the SARS-CoV-2 protease enzyme. This can thus be used to create a SARS-CoV-2 antiviral medication that is effective (Abd El-Mordy *et al.*, 2020).

2.3.5 Quinones

A group of chemicals with the quinone structure are known as quinones and result from the oxidation of hydroquinones (Alibi *et al.,* 2021). They can be found in higher species like insects, fungi, lichens, bacteria, and algae. Depending on the amount of benzene rings in the structural skeleton, it can be classified as benzoquinone, naphthoquinone, phenanthrenequinone, and anthraquinone (Lu *et al.,* 2013).

In benzoquinones, the saturated hexacyclic aromatic ring structure has two carbonyl groups. Naphthalene nuclei contains two carbonyl groups on a single nucleus which are used to identify naphthoquinones. Anthraquinones have the anthracene nucleus with two carbonyl groups, whereas polyquinones are dimers of the various quinones (Alibi *et al.*, 2021).

Quinones have both antibacterial and anticancer properties. For example, *Juglans mandshurica Maxim* and *Plumbago pearsonii L.* Bolus' have been isolated juglone and plumbagin respectively (Lu *et al.,* 2013).

Anthraquinones have a bacteriostatic effect on a variety of bacteria including *Pseudomonas aeruginosa, Corynebacterium pseudodiphtheriticum* and *Bacillus anthracis* (Alibi *et al.,* 2021). Naphthoquinones also have a significant antibacterial effect against *E. coli*, *S. aureus*, *P. aeruginosa*, *L. monocytogenes* as well as *Klebsiella pneumonia* (Ravichandiran *et al.,* 2019).

Quinones present in *Lawsonia inermis* have been documented to have antibacterial activity against *P. aeruginosa* (Nigussie *et al.,* 2021). *Annona squamosa* seeds, which have quinones in abundance, have been found to have potent antibacterial effect against *E. faecalis, S. aureus, P. aeruginosa, E. coli,* and *E. faecalis* (Nasser *et al.,* 2017).

2.3.6 Saponins

Natural soap-like substances known as saponins are steroidal or triterpenoid (Figure 2.4) generated by some bacteria, lower marine creatures and plants (Marrelli *et al.,* 2016). They are divided into steroidal saponins which are mainly abundant in monocotyledons and triterpenoid saponins which are predominant dicotyledons (Figure 2.4). They are known as saponins because they may create stable foams that resemble soap in aqueous solutions resulting from the combination of hydrophobic or fat-soluble sapogenin and hydrophilic sugar part (Marrelli *et al.,* 2016).

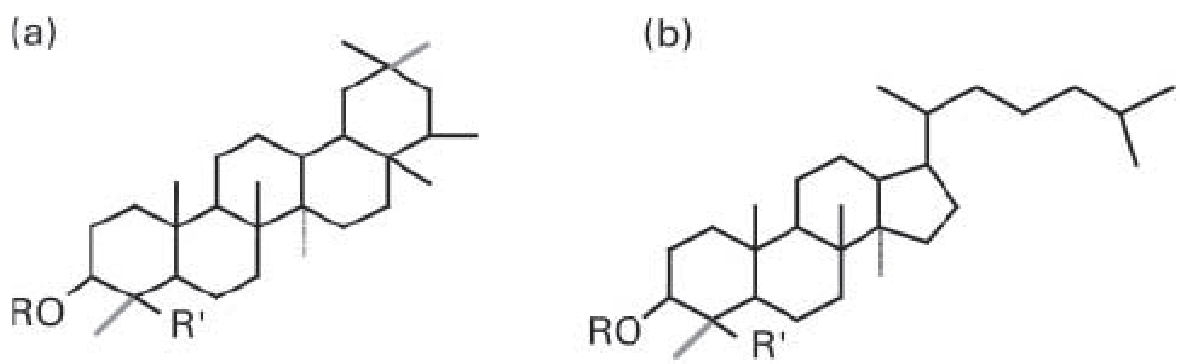

Figure 2.4: Basic structure of (a) triterpenoid and (b) steroidal saponins (Marrelli *et al.,* 2016).

Centella asiatica synthesizes secondary metabolites triterpenoid saponins during its normal growth and development. The active components of *C. asiatica* have demonstrated therapeutic advantages in the management of chronic venous diseases and problems with wound healing (Prakash *et al*., 2017; Pandey *et al*., 2020).

Saponins display a wide range of pharmacological activities, including expectorant, anti-inflamatory, anticancer, vasoprotective, gastroprotective, antimicrobial properties, insecticidal, molluscicidal, cytotoxic, pro-apoptotic, anti-invasive effects as well as exhibiting cholesterol lowering action in animals and humans (Koczurkiewicz *et al.*, 2015).

Saponins which have been isolated from roots of *Cassia auriculata* have been documented to be active against *Streptococcus faecalis*, *Aeromonas hydrophilia*, *Pseudomonas aeruginosa*, *Serratia ficaria*, *Salmonella typhae*, *Staphylococcus aureus* and *Escherichia coli* (Deshpande *et al.*, 2013).

Pharmaceutical enterprises are very interested in saponins since they are one of the components used in the semi-synthesis of steroidal medications (Zang *et al*., 2013). However, they can be toxic if given intravenously causing haemolytic activity on human erythrocytes as well as leading to gastroenteritis, diarrhea or even liver and/or kidney damage in ruminants (da Silva Marrelli *et al.*, 2016).

2.3.7 Steroids

Steroids are cholesterol derived lipophilic, low-molecular weight compounds with a characteristic structure of three C6-rings and a C5-ring, which are commonly linked to an alkyl side chain (position 17) and methyl groups (positions 10 and 13) (Figure 2.5) (da Silva Marineli *et al.*, 2015).

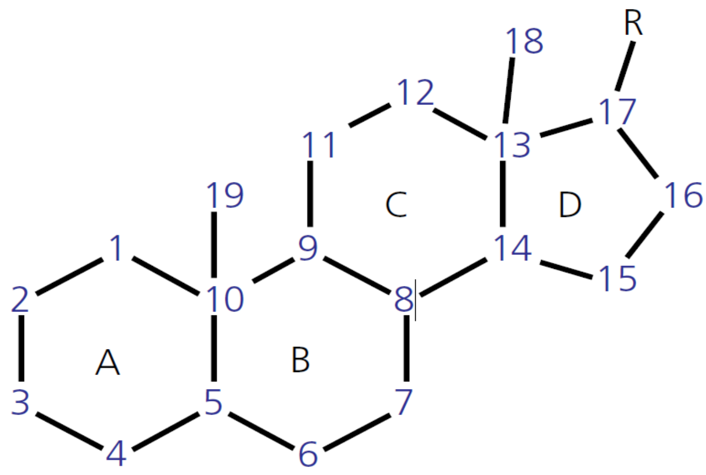

Figure 2.5: Basic carbon skeleton of steroids (da Silva Marineli *et al.*, 2015).

The use of synthetic steroids in antibacterial, anesthetic, anti-hormone, contraceptive, anti-cancer, anti-inflammatories, cardiovascular, osteoporosis, and anti-asthmatic medications is widespread (Sultan *et al.*, 2015).

Steroids can be categorized depending on a variety of factors, including their chemical makeup, place of synthesis (ovarian or adrenal steroids), biological purpose (glucocorticoid or sex steroids), and biochemical or molecular functions (Sultan *et al.*, 2015).

2.3.8 Tannins

Tannins are complex polymers or oligomers of polyphenolic chemicals that are found in nature and have high enough molecular weight to bind to proteins. Based on the structural characteristics, they may be divided into hydrolysable tannins and condensed tannins (Figure 2.6). Condensed tannins (Figure 2.6 a), also known as proanthocyanidins, are formed from flavonoid monomers, while hydrolysable tannins (Figure 2.6 b) are many carbohydrate-containing esters of gallic acid (Alibi *et al.*, 2021).

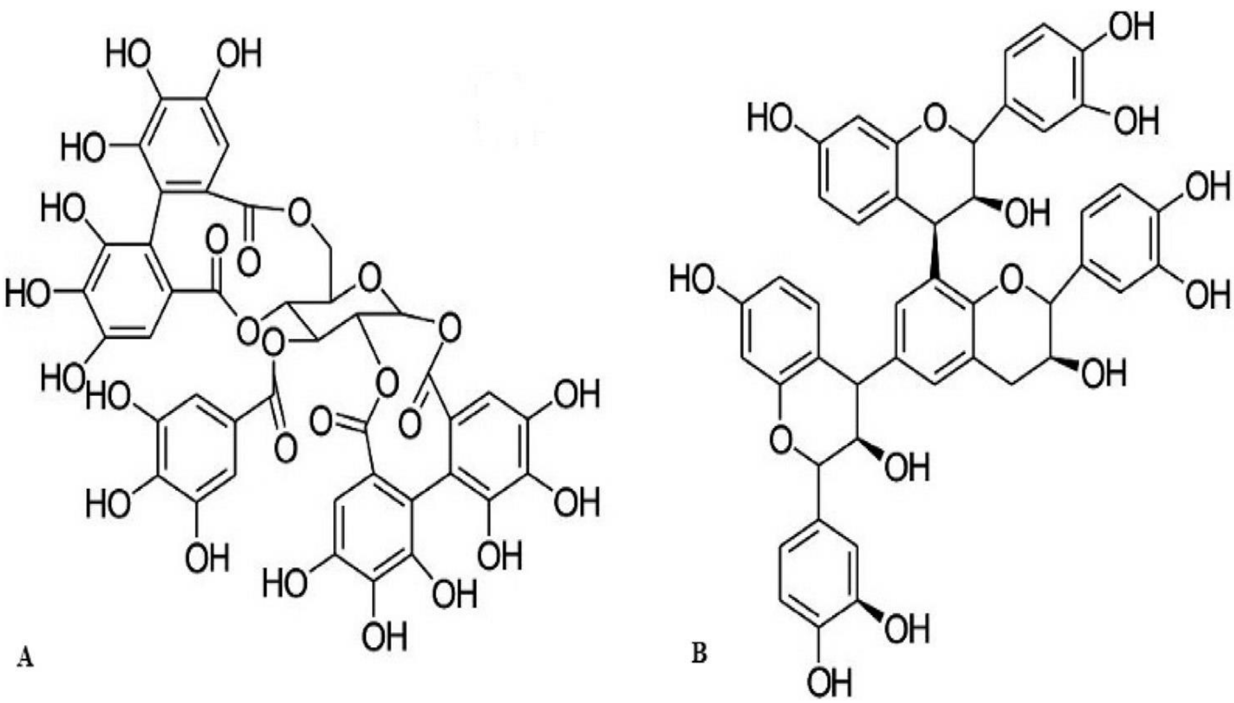

Figure 2.6: Structure of (A) hydrolysable tannins and (B) condensed tannins (Alibi *et al.*, 2021).

The ester bond's hydrolysis has been linked to the hydrolysis of hydrolysable tannins' antibacterial properties, and they exhibit strong synergies with antibiotics. They however have low pharmacokinetic property (Ekambaram *et al.*, 2016). *Ephedra sinica* stems contain concentrated tannins that are effective against methicillin-resistant *Staphylococcus aureus* (MRSA) and *P. aeruginosa* (Zang *et al.*, 2013). Condensed tannin-rich cranberry extract decreases *P. aeruginosa* virulence and prevented quorum sensing. The cranberry product Ellura, which is high in proanthocyanidins, is used to prevent urinary tract infections (UTIs) (Alibi *et al.*, 2021).

2.3.9 Terpenoids

Terpenoids are derivatives of terpenes or modified terpenes by oxidation or/and rearrangements. Terpene are biosynthetically synthesised from isoprene (2-methylbutadiene) molecule having a carbon skeleton (Figure 2.7) (Njeru *et al.,* 2013). Terpenes have multiples of C_5H_8 as their fundamental molecular formula. Terpenes are cyclic compounds that are categorized according to the quantity of C_5 isoprene units they contain. They can be hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}), carotenoids (C_{40}) and polyterpenes which have long chains made of many isoprene units (Alibi *et al.,* 2021).

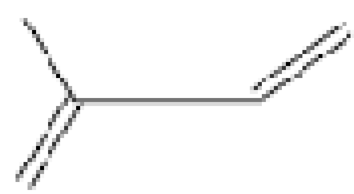

Figure 2.7: Isoprene unit (Njeru *et al.,* 2013).

Many different types of terpenes and terpenoids are present in a wide range of medicinal plants and have anti-bacterial, antiviral, antiprotozoal, anti-allergens, antiseptic as well as antifungal properties (Mishra *et al*., 2013). Terpenoid carvacrol is significantly active in controlling human pathogenic bacteria especially food-borne microorganisms such as *E. coli*, *Bacillus cereus* and *Salmonella* species (Alibi *et al.,* 2021).

Triterpenic acids like Asiatic, Centellic, and Madecassic acids are among the terpenoids that have the most medical potential. Terpenoids also include asiaticoside, centelloside, madecassoside, and centellose. Centelloids, also known as pentacyclic triterpenoid saponins (up to 8%), are the most significant compounds identified from *C. asiatica* (Kunjumon *et al.,* 2022). The glycosides Asiaticoside, madecassoside, and centelloside have been identified and are responsible for its pharmacological benefits in addition to being rich in terpenoids and flavonoids (Roy *et al.,* 2013; Prakash *et al.,* 2017). The fatty oils consist of glycerides of palmitic, stearic, lignoceric, linoleic, linolenic and oleic acids (Singh, 2015).

2.4 Antimicrobial activity

Chemicals known as antimicrobial agents either stop or eliminate bacterial development. (Idris & Nadzir, 2017). Over the years, numerous antibiotics and antimicrobial medications have been created to enhance human quality of life. However, improper antibiotic usage has rendered bacteria resistant (Anand *et al.,* 2019).

Antimicrobial resistance refers to the development over time of bacterial, viral, parasitic, and fungal germs that are resistant to antibiotics that were once utilized to treat infections (O'Neill *et al.,* 2016). Utilization of plant extract as antibacterial agents is encouraged by the undesirable side effects of several antibiotics (Ventola, 2015: Li *et al.,* 2017). *C. asiatica*, *D. repens* and *H. mannii* are some of the plants used extensively for medicinal purposes. Several phytochemical components having antimicrobial properties have been isolated and identified from such plants. Secondary metabolites of plants are well known for their properties such as larvicidal, bactericidal and fungicidal activities (Khalid *et al.*, 2018).

2.4.1 Antibacterial activity

Medicinal plants have antibacterial activity against various bacteria (Prakash *et al.*, 2017; Zahara *et al.*, 2021). Gram negative bacteria (*Shigella dysenteriae*, *Escherichia coli*, *S. paratyphi*, *S typhi*, *P. aeruginosa*, *Shigella boydii*, *Vibrio mimicus* and *V* *parahemolyticus*) as well as gram positive bacteria (*Bacillus megaterium*, *B. cereus*, *B. subtilis*, *S. aureus* and *Sarcina lutea*) were negatively affected by carbon tetrachloride, n-hexane and methanol extract from *C. asiatica* (Zahara *et al.*, 2021). Diethyl ether, dichloromethane, hexane, ethyl acetate and methanol extracts of *C. asiatica* inhibit *K. aerogenes*, *P. vulgaris*, *B. subtilis* and *S. aureus* (Zahara *et al.*, 2021). Both methicillin-resistant *S. aureus* and gram-positive *S. aureus* ATCC 25923 are susceptible to the antibacterial effects of *C. asiatica* methanol extract (Prakash *et al.*, 2017). 95% ethanolic extract of *C. asiatica*, *Chrysanthemum indicum* and *Andrographis paniculata* had a minimum inhibitory concentration of 16 µl/ml against *B. cereus* and 8 µl/ml for *L. monocytogenes* (Prakash *et al.*, 2017). *B. cereus*, *E. coli*, *S. aureus* and *P. aeruginosa* are all completely inhibited by methanol extracts of *C. asiatica* leaves (Anand *et al.*, 2019).

2.4.2 Antifungal activity

Centella asiatica extracts from petroleum ether and ethanol had significant antifungal action against *Candida albicans*, *Aspergillus flavus* and *A. niger* (Zahara *et al.*, 2021). *Saccharomyces cerevisiae*, *Aspergillus niger* and *Candida albicans* are inhibited by carbon tetrachloride, Chloroform, Hexane and aqueous soluble fractions of methanolic ethanolic extracts of *C. asiatica* (Zahara *et al.*, 2021). *Aspergillus flavus* and *Penicillium citrinum* showed a substantial mycelial inhibition of 26.3 mm when exposed to *C. asiatica* ethanolic extract (Dhiman *et al.*, 2016). *C. asiatica*'s 100% ethanolic extract displayed a 15.4 mm zone of inhibition against *A. niger* (Idris & Nadzir, 2017). Antimicrobial activity of ethanolic extract *of C. asiatica* had an inhibition of 16 and 15 mm on *A. niger* and *Candida albicans* respectively, with ketoconazole (10 µg) which was used as a control giving an inhibition zone of 10 mm (Prakash *et al.*, 2017).

2.4.3 Antiviral activity

Water extract of *C. asiatica* along with extracts of other plants like *Mangifera indica* combined are active against herpes simplex virus. Aqueous extract of *C. asiatica* showed increased antiviral activity against type 2 Herpes simplex virus (Roy *et al.,* 2013; Zahara *et al.,* 2021).

2.4.4 Antiprotozoal activity

Entamoeba histolytica is sensitive to alcoholic extracts of *Centella asiatica* (Singh, 2015). In dogs naturally infected with *Dirofilaria immitis*, a blend of ethanolic extracts of *C. asiatica* and *Acacia auriculi formis* significantly lowers filarial levels (Zahara *et al.*, 2021). Yucca saponins have been documented as effective against protozoan *Giardia lamblia* (Njeru *et al.*, 2013).

2.4.5 Larvicidal activity

Methanol, chloroform, hexane, ethyl acetate and acetone extract of *Centella asiatica* inhibited *Anopheles subpictus*, *Culex tritaeniorhynchus*, *Paramphistomum cervi*, the sheep fluke, and *Haemaphysalis bispinosa*, the adult bovine tick. However, *P. cervi* and *A. subpictus* respond best to methanol extract (Roy *et al.*, 2013).

2.5 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 (Bahadur *et al.,* 2015). 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). Plants having allelopathic traits can compete with weeds that are grown alongside agricultural crops, which can hinder crop development and production (Casimiro *et al.,* 2017). Weeds are a wide variety of economically useless plants that either grow alongside crops or in areas where they are not needed, resulting in economic losses (Anwar *et al.*, 2019). They are a threat to crop production causing a reduction in yields both qualitatively and quantitatively as a result of competition and/or allelopathy (Anwar *et al.*, 2019).

Cereals are any grass(s) cultivated for its edible grains which are an important part of diet in almost every meal worldwide. Their production faces yield declines due to weeds, soil toxicity and soil sickness (Zohaib *et al.,* 2016). Allelochemicals liberated from the weeds are likely to influence germination of seeds (Pélagie Michelin *et al.,* 2016), seedling growth, development and establishment and is regarded as a crucial component of an invasive species' success in natural and agricultural habitats (Kimura *et al.*, 2015). Since the vegetative growth of plants substantially influences the reproductive growth, a healthy balance between reproductive vegetative growth is necessary to achieve a good yield (Zohaib *et al.,* 2016).

Traditional field crops' germination and growth have been documented to be adversely affected by medicinal plants (Nazir *et al.,* 2014). Their impact increases with increased leaf extracts concentration. Extracts of *Melia azedarich* L. affects seed germination and seedling growth of *Penisitum americanum* L. (Bibi *et al.*, 2016). Allelopathic effects of kalmegh leaf extract on wheat seed germination and seedling growth has also been documented (Mandal *et al.,* 2016). Anwar *et al*. (2019) also documented allelopathic potential of *Carica papaya* against wheat crop. Evaluation of aqueous extracts of *Chenopodium album L.* for wheat (*Triticum aestivum L.*) yield and growth has also been documented (Majeed *et al.,* 2012). Rice seed germination, shoot length, root length, fresh and dry weight are all inhibited by *Centella asiatica* leaf and stem extracts, with leaf extracts being more detrimental than stem extracts (Alagesaboopathi, 2018). Extracts of Peanut (*Arachis hypogaea* L.) have also been documented to possess allelopathic activity on Lettuce (*Lactuca sativa*) (Casimiro *et al*., 2017).

2.6 Otitis Media

Otitis media (OM) is inflammation of the middle ear that is attributed to ear infections and is usually characterized by production of mucins in the middle ear mucosa (Qureishi *et al.,* 2014). Ear infections are more common in kids between the ages of 6 to 24 months and 3-5 years due to exposure to environmental factors while attending day-care and/or Kindergarten (Qureishi *et al.,* 2014). By the time they are seven years old, up to 60% of children are likely to have had at least one case of otitis media (Todberg *et al.,* 2014). In children, ear infections may lead to hearing loss and delayed speech (Yiengprugsawan & Hogan, 2013).

The main causes of hearing loss are fluid present in the middle ear or a tympanic membrane rupture. According to the World Health Organization, 328 million adults as well as 32 million children worldwide have mild hearing loss resulting from otitis media which is common in males than in females (WHO, 2015; Argaw-Denboba *et al.,* 2016). The three basic types of otitis media are acute, chronic suppurative, and otitis media with effusion (Schildler *et al.,* 2016).

2.6.1 Acute Otitis Media

An ear infection with a quick onset known as acute otitis media (AOM) typically begins with ear pain, decreased appetite and a fever may also be present (Lieberthal *et al.*, 2013).

AOM affects about 11% of world’s population each year. More than half of these cases involve children under five years of age with majority being males (Monasta *et al.*, 2012). It has to do with Down syndrome, bacterial infections, immune system development in children, and childhood anatomy (Minovi & Dazert, 2014).

2.6.2 Otitis Media with Effusion

Otitis medium with effusion (OME) is characterized by the accumulation of non-infectious fluid in the middle ear and mastoid air cells as a result of the negative pressure created by the Eustachian tube's failure for more than three months (Minovi & Dazert, 2014). OME is also known as serous otitis media (SOM), secretory otitis media (SOM), or simply 'glue ear,' (Schildler *et al.,* 2016). OME occurs following AOM and is associated with viral upper respiratory infections, bacterial infections, irritants or allergies. The most common symptom of OME is an occasionally reported sense of fullness (Lieberthal *et al.,* 2013; Minovi & Dazert, 2014).

2.6.3 Chronic Suppurative Otitis Media

Middle ear and mastoid cavity chronic inflammation known as chronic suppurative otitis media (CSOM), characterized by a ruptured tympanic membrane that permits fluid to flow from the middle ear for longer than a month (Prakash *et al.,* 2013; Emmett *et al.*, 2018). CSOM affects about 5% of the human population annually with 22.6% of these cases occurring in children under the age of five years (Monasta *et al.,* 2012). According to WHO, about 4% of the world’s population having AOM can develop CSOM causing approximately 21,000 deaths as a result of complications of CSOM every year (Prakash *et al.,* 2013; Schildler *et al.,* 2016).

2.6.4 Microbiology of Otitis Media

Causal agents of ear infections can be viral, bacterial or a combination of the two (Marom *et al.,* 2012; Schildler *et al.,* 2016; Khattak *et al.,* 2017; Emmett *et al.,* 2018). Bacteria like *Haemophilus influenzae*, *Proteus species*, *Pseudomonas species*, *Escherichia coli, Citrobacter species, Enterobacter species, Klebsiella species, Staphylococcus epidermidis* and *Staphylococcus aureus* have been isolated from the middle ear both individually and as complexes (Argaw-Denboba *et al.,* 2016).

According to Afolabi *et al.* (2012) and Khattak *et al.* (2017), ear infections are caused by *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *E. coli*, *S. aureus*, *Proteus mirabilis* and *Klebsiella* species. However, Saraca *et al.* (2019) found that the bacteria that frequently cause otitis media are *S. pneumoniae, H. influenzae* and *M. catarrhalis,* with *P. aeruginosa* and *S. aureus* associated with the chronic form of otitis media.

Influenza viruses (types A and B), parainfluenza viruses (types 1, 2 and 3), Adenoviruses, respiratory syncytial virus (RSV), enterovirus, rhinovirus and coronavirus are among the viruses that are frequently linked to otitis media (Qureishi *et al.,* 2014). Some viruses like Influenza A virus, respiratory syncytial virus (RSV) and coronaviruses do not directly cause otitis media, but they may increase the risk of middle ear infections that result in inflammation (Marom *et al.,* 2012; Emmett *et al.,* 2018).

2.6.5 Management and prevention of Otitis Media

Management of otitis media involves the use of pain-relieving medications as well as antibiotics for those who are severely ill or are under two years old (Lieberthal *et al.,* 2013; Qureishi *et al.,* (2014). 80% of acute otitis media cases settle without treatment meaning that medication is recommended only if pain is present. Acetaminophen (paracetamol), ibuprofen, benzocaine ear drops, and opioids are some examples of painkillers (Qureishi *et al.,* 2014). Amoxicillin is the initial antibiotic of choice and speeds recovery from acute otitis media as well as increasing resolution of symptoms for cases of otitis media with effusion. However, Children with otitis media with effusion may experience diarrhea, vomiting, and skin rashes as a result of it (Venekamp *et al.*, 2016). The primary antibiotic of choice is amoxicillin, but if resistance develops, amoxicillin-clavulanate or any other another penicillin derivative combined with a beta lactamase inhibitor can be used (Lieberthal *et al.,* 2013). Administration of Pneumococcal conjugate vaccines (PCV), vaccination against influenzas well as avoiding tobacco smoke have significantly decreased the risk of otitis media (Lieberthal *et al.*, 2013).

2.7 Bacteria of interest

Bacteria are microbial pathogens which can be beneficial or can be agents responsible for infectious illnesses with a high mortality rate that are of grave concern for public health particularly if they acquire resistance to therapeutics (Varela *et al.*, 2021). *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* are among bacteria which have been documented as the major causative agents of ear infections in Ethiopia (Argaw-Denboba *et al.,* 2016).

2.7.1 *Escherichia coli*

Escherichia coli is gram negative bacteria in the *Enterobacteriaceae* family. *E. coli* is a non-sporulating facultative anaerobe. A little more than 80% of instances of meningitis and 40% of cases of septicemia are caused by *E. coli* strains that produce the K1 capsular polysaccharide antigen. It has also been associated with ear infections (Makvana & Krilov, 2015; Argaw-Denboba *et al.*, 2016). Numerous diverse diarrheal diseases are linked to various *E. coli* strains. 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). E. coli acts as a substantial reservoir for transmissible antibiotic resistance because it commonly harbors drug-resistance plasmids and quickly transfers those plasmids to other species when stressed (Makvana & Krilov, 2015).

2.7.2 *Pseudomonas aeruginosa*

One of the main organisms responsible for nosocomial infections, *Pseudomonas aeruginosa* mainly affects patients with compromised immune systems or those hospitalized to the intensive care unit. It has also been associated with otitis media (Ruiz-Garbajosa & Canton, 2017; Mohammed & Abdullah, 2020). *P. aeruginosa* is a member of the *Pseudomonadaceae* family and is a non-fermenting gram-negative rod. It can occupy a wide range of ecological niches, but damp habitats in particular. It may acquire resistant genes horizontally or through chromosomal alterations to become resistant. Additionionally they can be intrinsically resistant to many antimicrobials causing bacteremia, severe pneumonia or even deaths (Argaw-Denboba *et al.*, 2016; Ruiz-Garbajosa & Canton, 2017; Mohammed & Abdullah, 2020).

2.7.3 *Staphylococcus aureus*

Staphylococcus aureus is a aerobic and facultative anaerobic gram positive bacilli of the family *Staphylococcaceae*. This catalase and coagulase positive commensal colonizes

30% of healthy individuals from different body parts like the anterior nares' skin and mucous membrane, as well as the perineum, genitourinary tracts, and even the pharynx (Mohammed & Abdullah, 2020). *S. aureus* also contributes to a variety of illnesses in humans and animals that have a big impact on public health. It plays a significant role in causing infections in humans and animals both in hospitals and the community ranging from simple to life threating infections. It has also been associated with otitis media with methicillin resistant *S. aureus* (MRSA) and Vancomycin resistant *S. aureus* (VRSA) having potential to cause epidemics worldwide (Argaw-Denboba *et al.*, 2016; Mohammed & Abdullah, 2020). They are a significant contributor to hospital-acquired infections that are increasingly resistant to all kinds of available antibiotics (WHO, 2018; Rasheed & Hussein, 2021).

2.8. 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 absence of efficient and effective antimicrobial drugs renders common infections to becoming more difficult to treat and individuals remain sick for a longer time hence long hospital stays and increase in morbidity and mortality (O'Neill *et al.*, 2016).

Antimicrobial resistance is as a result of three major factors that includes humans allowing dangerous pathogens into the environment, increase in the frequency of antimicrobial resistance phenotypes among microorganisms as well as the broad and usually unnecessary usage of antibiotics causing selective pressure pushing microorganism evolution (Michael *et al.*, 2014).

Virtually all microorganisms can acquire resistance to target antimicrobials at any time. Bacteria acquire resistance to antibiotics, fungi acquire resistance to antifungals, viruses acquire resistance to antivirals and protozoa acquire resistance to antiprotozoal antimicrobial agents (Anand *et al.,* 2019).

Resistance to antimicrobials can be multi-drug resistance, extensive drug resistance or pan-drug resistance. The acquired inability to respond to at least one antimicrobial agent from three or more antimicrobial classes is known as multi-drug resistance (MDR). Pan-drug resistance (PDR) is the acquired non-susceptibility to all antimicrobial agents in all classes, as opposed to extensive drug resistance (XDR), which is the acquired non-susceptibility to at least one antimicrobial agent in all but one or two antimicrobial classes (Sweeney *et al.,* 2018).

2.9. Antibiotic Resistant Bacteria (ARB)

Administration of antimicrobials for treatment and prevention of infections over time has provoked an evolutionary response among microorganisms to resist applied antimicrobial agents. Misuse and/or overuse of antibiotics can therefore result in bacteria acquiring resistance through selective pressure in the habitats they exist in (Michael *et al.,* 2014; Serwecińska, 2020).

Antibiotic-resistant microorganisms exists naturally occurring in the environment. Reservoirs and habitats of antibiotic resistant bacteria may include the skin, upper respiratory system and human and animal gastrointestinal systems as well as water, raw meat, dairy products, soils, sewage and some plants (Serwecińska, 2020). Some bacteria may have antibiotic resistant genes or undergo mutations that improve their ability to survive when antimicrobial agents are present. Susceptible bacteria in turn can obtain antibiotic resistance through acquisition of antibiotic resistance genes from other bacterial cells or through via de novo gene mutations. Bacteria can acquire antibiotic resistance genes by horizontal gene transfer (HGT), even across cells from different genera or species. This is achieved through genetic components such transposons, integrons, prophages and plasmids (Fernandez-Lopez *et al.,* 2016).

Gram-negative bacteria are intrinsically resistant to some antibiotics such as vancomycin due to the properties of their cell walls. Some strains of *Pseudomonas aeruginosa* have been documented to be resistant to chloramphenicol, tetracyclines, trimethoprim and sulphonamides. *Klebsiella* spp. are also not susceptible to ampicillin (Ruppé *et al.,* 2015).

Gram-negative bacteria especially from the family *Enterobacteriaceae* as well as those of the genera *Acinetobacter* and *Pseudomonas* are of particularly high concern due to their resistance patterns according to the World Health Organization (WHO) and therefore there is need for the development of newer and more effective antimicrobial agents urgently (WHO, 2018; Serwecińska, 2020).

Gram positive bacteria like methicillin resistant *Staphylococcus aureus* (MRSA), Vancomycin resistant *S. aureus* (VRSA) and vancomycin-resistant *Enterococcus* (VRE) bacteria are also of major concern for public worldwide and have been implicated as having potential to cause epidemics (WHO, 2018; Rasheed & Hussein, 2021).

2.10. Bacterial Mechanisms of Resistance

Bacteria which are resistant to multiple antimicrobial agents can result in serious public health concerns and a buildup of highly infectious diseases. Bacterial resistance can be either maintained on the bacterial chromosome (intrinsic) or can be acquired through conjugation, transduction, mutations of the chromosome, transformation of plasmids and transposition. Multiple molecular and cellular pathways for resistance are present in multidrug-resistant bacteria (Varela *et al.*, 2021). Bacterial resistance strategies include limiting drug absorption and/or access into pathogen cells, enzymatic metabolism of antibiotics, the creation of biofilms, altering/modifying drug targets, and using active drug efflux pumps (Reygaert, 2018; Varela *et al.*, 2021).

2.10.1 Reduced uptake and/or entry

Certain types of chemicals can pass through the cell walls and membranes of bacteria thanks to their structure and function. *S. aureus* has been documented to have produced a thicker cell wall that makes it difficult for medications and other antimicrobials to enter the cells, developing resistance to vancomycin (Miller *et al.*, 2014).

Bacteria may simply stop antimicrobial substances from entering their cells by reducing drug permeability. Strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica and Vibrio cholerae* have been documented to utilize this mechanism in particular (Varela *et al.,* 2021). Bacteria like Mycoplasma which lack a cell wall are intrinsically resistant to antimicrobials like β-lactams which target the cell wall. The outer membrane's high lipid content facilitates the uptake of hydrophobic medications like rifampicin but prevents hydrophilic substances from passing through (Reygaert, 2018).

Porins are hydrophilic channels that mimic pores and allow specific sizes and charges of molecules to flow through. They are involved in the molecular mechanism for giving resistance through reduced permeability. Reduced drug uptake is achieved by decreasing the number of porins present as well as undertaking mutations that change the selectivity of the porin channel (Reygaert, 2018).

Wide bacterial porin can be highly selective and do not allow many antimicrobial agents to pass through (Varela *et al.,* 2021). Strains of *Pseudomonas aeruginosa* have a porin called OprD which can mutate reducing permeability of the outer membrane resulting in resistance to quinolones and aminoglycosides. Mutations leading to changes in porins has been observed in *Neisseria gonorrhoeae* and in *E. aerogenes* leading to resistance to tetracycline and cephalosporins respectively (Reygaert, 2018).

2.10.2 Enzyme based drug inactivation

Bacteria can synthesize enzymes that are capable of degrading antibiotics. This enzymatic inactivation mechanism of antibiotic resistance includes hydrolysis and transfer of functional groups (Varela *et al.,* 2021). By hydrolyzing a particular location in the -lactam ring structure, -lactamases render -lactam drugs inactive, preventing them from binding to their intended protein targets. Chromosomal β-lactamase genes are present in *Pseudomonas* spp, *Aeromonas* spp. and *Acinetobacter* spp. Plasmid β-lactamase genes are found in *Staphylococcus aureus*, *E. faecium* and *E. faecalis* (Reygaert, 2018). Enzyme mediated transfer of functional groups like acyl, nucleotidyl, glycol, thiol, ribosyl or phosphoryl groups in antibiotics renders macrolides, rifamycins, chloramphenicol and aminoglycosides ineffective on the bacteria (Varela *et al.,* 2021).

2.10.3 Active efflux pumps

Bacteria possess efflux pumps which are an integral part of bacterial physiological genes. Efflux pumps are chromosomally encoded and are involved in many functions like maintenance of homeostasis and expulsion of toxic products of metabolism. However, bacteria such as *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA) as well as Multi Drug Resistant *Mycobacterium tuberculosis* utilize these efflux pumps to expel antimicrobial products enabling bacterial survival (Varela *et al.*, 2021).

Pseudomonas aeruginosa utilises the MexAB-OprM pumps to expell chloramphenicol, β-lactams, tetracyclines and fluoroquinolones. Some *Staphylococcus* species use SMR pumps on erythromycin, ampicillin and tetracyclines while *E. coli* utilises EmeR pumps to expell erythromycin, vancomycin and tetracyclines (Reygaert, 2018).

2.10.4 Protection of antimicrobial targets

Bacteria can also protect antimicrobial targets using other specific factors. The mechanisms of the target protection mechanisms include direct antibiotic displacement, allosteric antibiotic removal as well as the restoration of target normal functions of antimicrobial targets despite the existence of a bound antibiotic by utilising the target protection proteins (TPPs) (Wilson *et al.*, 2020).

Direct antibiotic displacement occurs when the antibiotic is directly removed from the target's active site by target protection proteins, blocking the antibiotic's antimicrobial effects. The target protection proteins can also induce conformational changes to target thereby re-establishing its normal functioning despite the existence of the antibiotic-target complex. The target protection protein can also bind to the allosteric site of the target and induce conformation changes resulting in the removal of the antibiotic from the target's binding site, therefore rendering it inactive and/or ineffective (Wilson *et al.*, 2020).

2.10.5 Altering/modifying drug targets

Bacteria can alter drug targets on the cells preventing drugs from binding and resulting in resistance. Vancomycin-resistant enterococci (VRE) as well as Vancomycin-resistant Staphylococcus aureus (VRSA) both develop resistance to the antibiotic as a result of acquiring van genes, which alters the structure of peptidoglycan precursors and reduces vancomycin's ability to bind, eventually leading to a complete inability to bind (Reygaert, 2018; Rasheed & Hussein, 2021).

2.10.6 Biofilm formation

Biofilm is a thick, sticky matrix containing polysaccharides, proteins and DNA from the resident bacteria. They shield harmful bacteria from immune system assault, preventing antibacterial chemicals from getting to the bacteria and destroying them (Mah, 2012). As with *Pseudomonas aeruginosa* in the lungs, biofilms can contain a dominating organism or can include a diverse range of organisms, as is the case with the biofilm community of normal flora present in the gut (Reygaert, 2018). Most bacterial cells present in the biofilm are sessile meaning that antimicrobials targeting growing and/or actively dividing cells have little effect on bacterial cells in the biofilm. Horizontal transfer of genes is also highly likely to occur in biofilms because of the proximity of the bacterial cells thereby resulting in the transfer of antimicrobial resistance easily by the bacterial communities present in the biofilm (Van Acker *et al.,* 2014).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Site for this study

Plant leaves used in this study were collected from the County of Nandi. The County is located in the North Rift of Kenya and the headquarters is at Kapsabet town. It occupies an area of 2,884.4 kilometres square (km^2). According to the 2019 Kenya Population and Housing Census, the population was 885,711 with the majority belonging to the Nandi tribe although there exists a number of other Kenyan communities as well (nandi.go.ke).

The County borders Uasin Gishu County to the East and North, Kakamega County to the West, Kisumu County to the South, Vihiga County to the South West and Kericho County to the South East. Nandi County has six (6) constituencies (Aldai, Chesumei, Emgwen, Mosop, Nandi Hills, Tinderet) as well as thirty electoral wards (nandi.go.ke). Nandi County is unique jug-shaped in structure, located between longitudes 34°49' E & 35°27' E and extends northwards from the south where it is bound by the Equator to latitude 0°34'N.

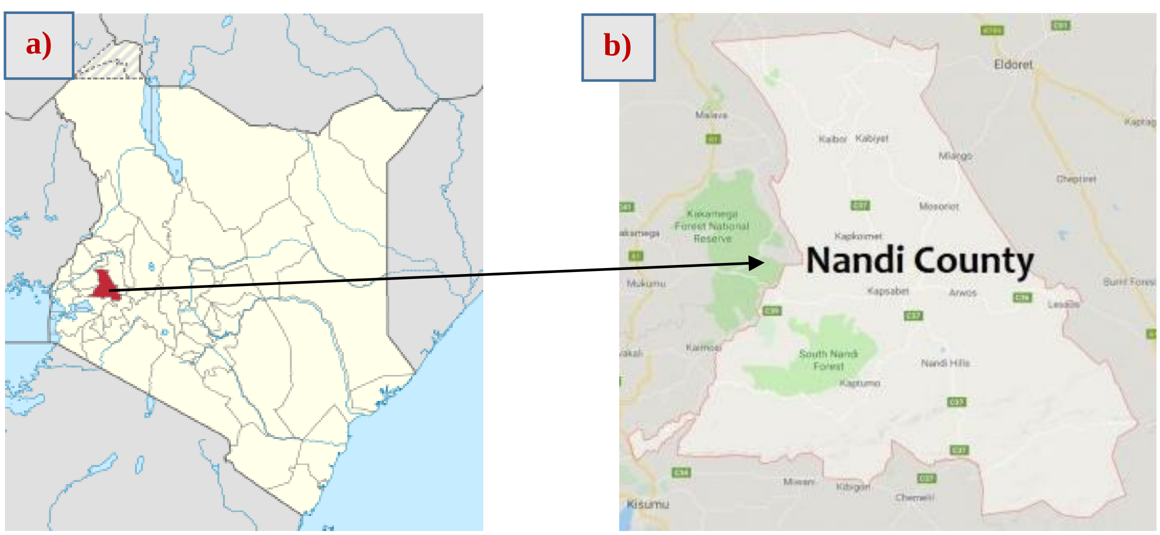

Figure 3.1: Map indicating geographical location of Nandi County (nandi.go.ke)

Nandi County has a cool and wet climate with temperatures ranging from a 12°C to 25°C and rainfall ranging from 1,200 to 2,000 millilitres per year. The county experiences two rainy seasons: lengthy rains from March to June and brief rains from September to November. There also exists fertile volcanic soils in the county making it an ideal area for farming tea, coffee, maize and sugar cane (nandi.go.ke).

3.2 Sample collection and storage

Centella asiatica, Dichondra repens and *Hydrocotyle mannii* grow as a weed community forming the *Hydrocotylo-Centelletum asiaticae* association. *C. asiatica* and *H. mannii* appears as the dominant species of the community (Mosango, 2017). This weed community is found growing in ruderal areas and cultivation areas which have been abandoned, particularly under shaded areas and in moist soils which are rich in organic matter forming dense mats near ponds, rivers, marshes ort on roadsides. *Dichondra repens* can also be found growing in more and/or less shady areas. These sites were identified in the fields within Nandi County. The leaves of *C. asiatica*, *D. repens* and *H. mannii* were identified and collected. The samples were placed in sterile sample collection containers, assigned codes C for *C. asiatica*, DR for *D. repens* and HM for *H. mannii*. they were then stored in cool boxes ready for transportation to University of Eldoret laboratory.

3.3 Preparation of plant samples

After collection, the plant leaves were hand washed in running tap water 2-3 times. Washing eliminated contamination from adhering particles such as any dirt or filthy particles present on the leaves surface. The washed plants were then dried to remove the water content from plants. The plants were weighed and then placed on stands to be air-dried in a shed to ensure that the plant's active compounds are not lost. 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 with unique codes and then stored in air tight sample collection containers and were transported to the laboratory for further analysis.

3.4 Extraction of the plant extracts

The powder from the plant leaves were each used for crude extraction. Water, ethyl acetate, diethyl ether and ethanol were used for extraction. One gram of sample to 4 ml of solvent (1:4) was the ratio used 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 at 55°C. 1g of the crude extract was dissolved into 1 ml of the respective solvents to make stock concentrations of 100 mg/ml. The percentage yield was then calculated using the formula below Felhi *et al.* (2017);

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

3.4.1 Aqueous extraction

Twenty-five grams of *C. asiatica*, *D. repens* and *H. mannii* crushed leaf powders were weighed and soaked in 100 ml double distilled water each. 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.

3.4.2 Extraction using Diethyl ether

Twenty-five grams of *C. asiatica*, *D. repens* and *H. mannii* crushed leaf powders were weighed, placed into separate 250 ml conical flasks and 100 ml of diethyl ether added into each flask. The mixtures were left to settle with continuous agitation in a shaker for 48 hours at room temperature. They were then filtered using Whatman's no. 1 filters and collected into clean conical flasks. Extracted solutions were concentrated using a rotary evaporator then stored in a fridge 4°C awaiting subsequent processes.

3.4.3 Extraction using Ethanol

Twenty-five grams of *C. asiatica*, *D. repens* and *H. mannii* crushed leaf powders were weighed, placed into separate 250ml conical flask and one hundred ml of ethanol was added to each sample then shaken well. 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 further use.

3.4.4 Extraction using Ethyl acetate

Twenty-five grams of *C. asiatica*, *D. repens* and *H. mannii* crushed leaf powders were weighed, placed into separate 250ml conical flask and 100 ml of ethyl acetate added to each sample. The samples were left to settle with continuous agitation in a shaker at room temperature for 48 hours, filtered using Whatman's no. 1 filters and then concentrated using a rotary evaporator. The filtrates were then sealed with sterile cotton wool and aluminium foil and stored in a fridge set at 4°C awaiting subsequent procedures.

3.5 Antibacterial bioassays of *C. asiatica*, *D. repens* and *H. mannii* plant extracts.

3.5.1 Culture media preparation

Media used for bioassays were nutrient agar and nutrient broth. Twenty-eight (28) grams of nutrient agar and thirteen (13) grams of nutrient broth were each suspended in 1000 ml of distilled water. Media was sterilized at 121^{o}C for 15 minutes in an autoclave, cooled to 40^{o}C before dispensing 15-20 ml into sterile petri plates and allowed to further solidify.

3.5.2 Microorganisms used and their preparation

Otitis media associated ATCC bacteria (*Escherichia coli* ATCC 25922, clinical isolate of *Pseudomonas aeruginosa* and *Staphylococcus aureus* ATCC 25923) obtained from Kenya Medical Research Institute (KEMRI) were used in the study. The ATCC strains and clinical bacterial isolates were sub-cultured onto nutrient agar and then incubated at 37^{o}C for 24 hours to obtain freshly grown bacterial strains. 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.

3.5.3 Susceptibility testing

In accordance with the Clinical and Laboratory Standards Institute's recommendations (CLSI, 2020) susceptibility tests were carried out utilizing the disc diffusion method with slight modification. Disc diffusion was done using nutrient agar media. The microbial cultures were inoculated using a sterile swab and distributed uniformly across the media's whole surface. 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^{-3}) was micro pipetted into the sterile filter paper discs and allowed to soak. Ciprofloxacin was used as a positive control with the respective solvents used as negative control. Discs with desired concentrations were placed aseptically onto plates with media and inoculated with sterile forceps and incubated at 37°C for 24 hrs. All experiments were conducted in three replicates.

The 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 according to Clinical and Laboratory Standards Institute (CLSI), 2020 guidelines.

3.5.4 Data handling and statistical analysis for antibacterial activity.

Antibacterial bioactivity of the extracts against *Escherichia coli* ATCC 25922, clinical isolate of *Pseudomonas aeruginosa* and *Staphylococcus aureus* ATCC 25923 was assessed using disk diffusion where the inhibition zone diameters were measured in millimeters and entered into Microsoft Excel 2019 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 multifactor analysis of variance (MANOVA).

3.6 Allelopathic activities of *C. asiatica*, *D. repens* and *H. mannii* leaf extracts.

3.6.1 Testing for allelopathic activity.

Approximately twenty-five grams of *C. asiatica*, *D. repens* and *H. mannii* crushed leaf powders were soaked in 100ml double distilled water, diethyl ether, ethanol and ethyl acetate 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), oats (S18), rice (NERICA L-19), sorghum (E-1291) and wheat (durum) obtained from Kenya seed company in petri dishes. Before the germination test, the seeds were first surface sterilized using 1% sodium hypochlorite (NaOCl) for 1 minute, then rinsed twice with double distilled water to remove excess chemicals. Petri dishes were prepared by cleaning, autoclaving and then lining them with a thin lining of sterilized serviettes.

Effects of the plant extracts on germination was tested by placing 10 surface sterilized seeds in each petri dish. Twenty-four millilitres of the test extracts from the experimental plants was then added to it. The petri dishes treated with distilled water was set as positive control with those with Dimethyl sulpoxide set as negative control. The experiments were laid out in a Completely Randomized Design (CRD) with three replications for each plant extracts and test seed species. The setup was then kept undisturbed in room temperature (25±2°C) in the laboratory. The emerged plumule and radicle lengths were measured after 5 days using a sterile string, transferred onto a ruler the measurements recorded. The number of seeds germinated was also counted after 5 days and the germination percentage calculated using the formula below:

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

3.6.2 Data handling and statistical analysis for allelopathic activity.

A database was created in Microsoft Excel 2019 where data was entered in separate spreadsheets. The effect of plant extracts on plumule and radicle lengths was determined using multifactor analysis of variance (MANOVA) and considered statistically significant at 95% confidence level ($P \leq 0.05$).

3.7 Qualitative analysis of phytochemicals in *Centella asiatica, Dichondra repens* and *Hydrocotyle mannii.*

For qualitative phytochemical analysis, the already prepared stock concentrations were used. The filtrates from all the solvents were used to test for the absence or presence of various phytochemicals according to the following described protocols;

3.7.1 Test for alkaloid (wagner's test)

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).

3.7.2 Test for coumarins

Two millilitres of 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).

3.7.3 Test for flavonoids (alkaline reagent test)

In a test tube, 5 millilitres of dilute ammonia solution was added to 1 millilitre of extract and 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).

3.7.4 Test for glycosides (borntrager's test)

Three millilitres of chloroform was added to two millilitres of plant extract in a test tube and shaken. Chloroform layer was separated using a pipette and 10% ammonia solution was added. Presence of glycosides was confirmed by appearance of a pink colour (Balamurugan *et al.,* 2019).

3.7.5 Test for phenols (lead acetate test)

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

3.7.6 Test for quinones

One millilitre of alcoholic 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).

3.7.7 Test for steroids

A 2ml sample of chloroform was added to 2 ml of extract in a test tube. The mixture was then agitated after 2ml of strong sulfuric acid was added. The appearance of red colour and yellowish green fluorescence indicated the presence of steroids (Balamurugan *et al.,* 2019).

3.7.8 Test for saponins (foam test)

In a test tube, 2 millilitres of extract was drawn then mixed thoroughly with 2 millilitres of distilled water then shaken vigorously and warmed. A few drops of olive oil was then added, 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).

3.7.9 Test for tannins (ferric chloride test)

Five millilitres of the filtered extract was pipetted into a test tube. Three drops of 5% ferric chloride solution was then gently added. The production of a dark green colour showed that tannins were present in the sample (Balamurugan *et al.*, 2019).

3.7.10 Test for terpenoids (salkowski's test)

One millilitre of chloroform was mixed with two millilitres of the sample in a test tube. Three drops of concentrated sulphuric acid was then carefully added through the sides to forming a layer. Appearance of a reddish-brown colour at the interface was indicative that terpenoids were present in the sample (Balamurugan *et al.*, 2019).

CHAPTER FOUR

RESULTS

4.1 Percentage yields of plant samples

Percentage yield was calculated as described by Felhi *et al.* (2017) for each powdered plant material on each of the solvents used after the extraction process. In general, the mean percentage yield achieved after extraction was 14.65% for *D. repens*, 14.21% for *C. asiatica* and 13.88% for *H. mannii* (Table 4.1). The highest percentage yield was achieved from aqueous extract of *C. asiatica* at 16.24% while the lowest percentage yield was the extract of *H. mannii* extracted using diethyl ether at 12.98% as shown in Table 4.1. This indicates that extraction favours solvents with a higher polarity (Truong *et al.*, 2021).

Table 4.1: Percentage yields after extraction

Plant	Solvent	% yield	Mean % yield
Centella asiatica	Diethyl ether	13.23	
	Ethanol	13.97	
	Ethyl acetate	13.39	14.21
	Water	16.24	
Dichondra repens	Diethyl ether	13.4	
	Ethanol	15.29	
	Ethyl acetate	14.16	14.65
	Water	15.74	
Hydrocotyle mannii	Diethyl ether	12.98	
	Ethanol	13.42	
	Ethyl acetate	13.49	13.88
	Water	15.62	

4.2 Efficiency of solvents used for extraction

Water, diethyl ether, ethyl acetate and ethanol solvents were used for extraction process with results showing that each solvent had different efficiency irrespective of the plant as is justified by the percentage yields obtained (Table 4.1). Water produced the highest crude extract mean percentage yield of 15.87% while diethyl ether had the least (13.2%). Ethanol and ethyl acetate had 14.23% and 13.68% respectively (Figure 4.1).

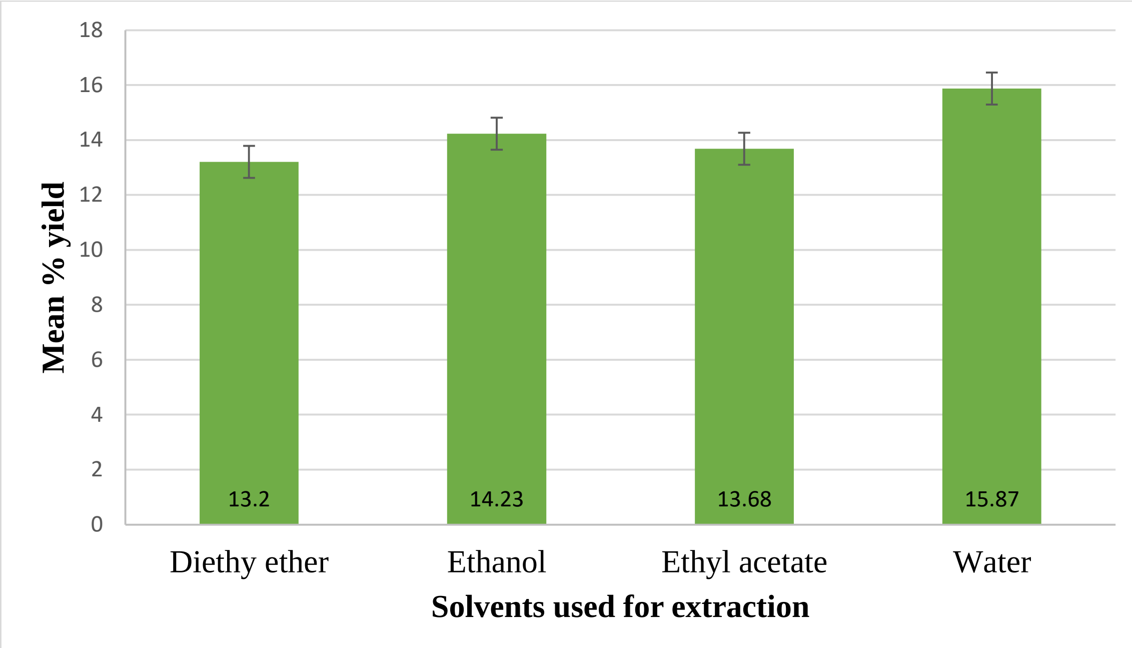

Figure 4.1: Mean percentage yield of the different solvents used for extraction.

4.3 Antibacterial activities of crude extracts

Extracts of *C. asiatica*, *D. repens* and *H. mannii* on average had almost similar antibacterial effect on the test bacteria. Ethanolic extracts showed a higher antibacterial activity compared to aqueous, diethyl ether and ethyl acetate extracts. The extracts in all the concentrations (10^0 to 10^{-3}) were more active against *Staphylococcus aureus* ATCC 25923 followed by *Escherichia coli* ATCC 25922 and little activity on the clinical isolate of *Pseudomonas aeruginosa*. Antibacterial activities of *C. asiatica*, *D. repens* and *H. mannii* extracts against *E. coli* ATCC 25922, clinical isolate of *P. aeruginosa* and *S. aureus* ATCC 25923 are presented in Figures 4.2, 4.3 and 4.4 respectively.

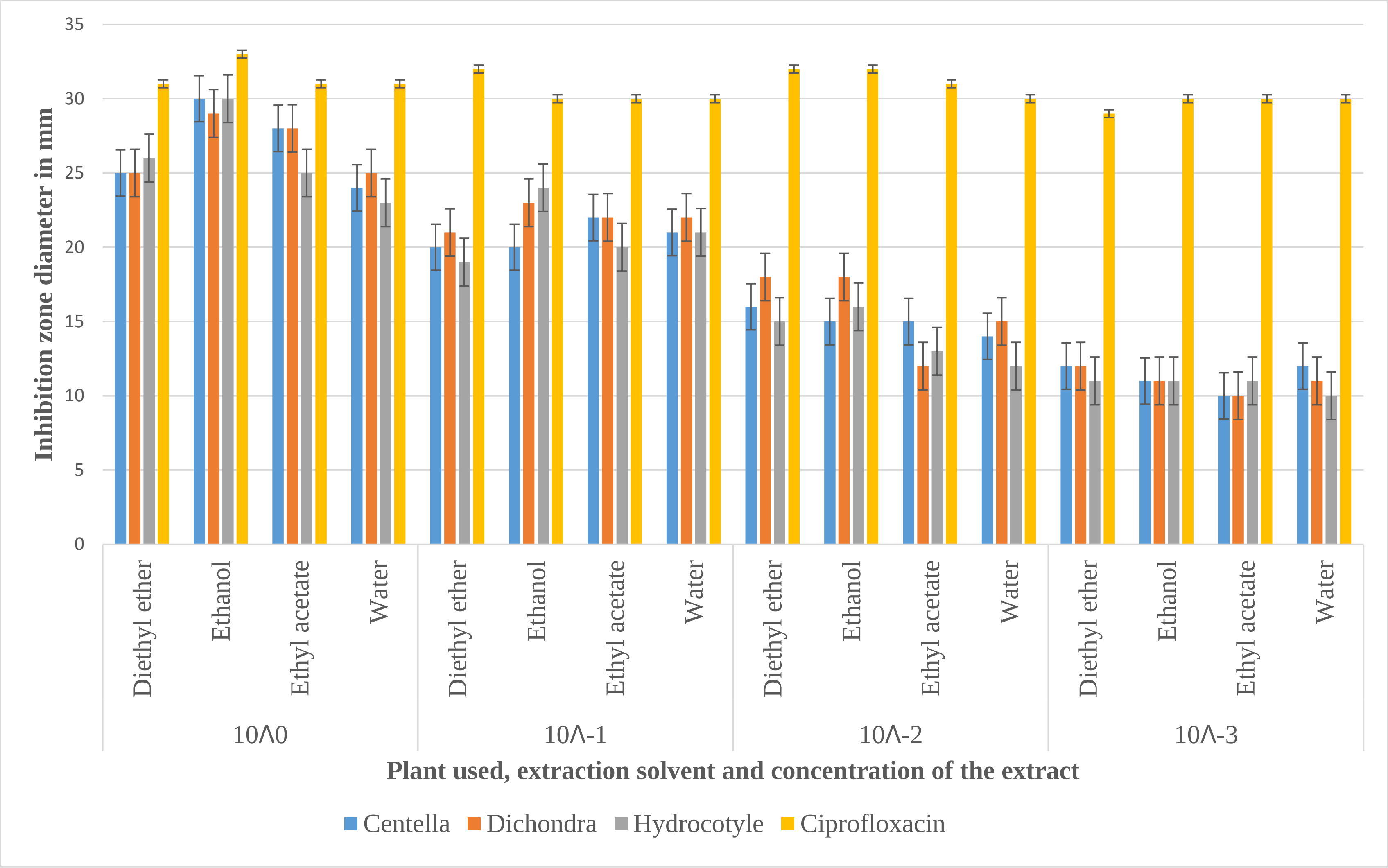

Figure 4.2: Antibacterial activities of *C. asiatica, D. repens* and *H. mannii* against *Escherichia coli* ATCC 25922.

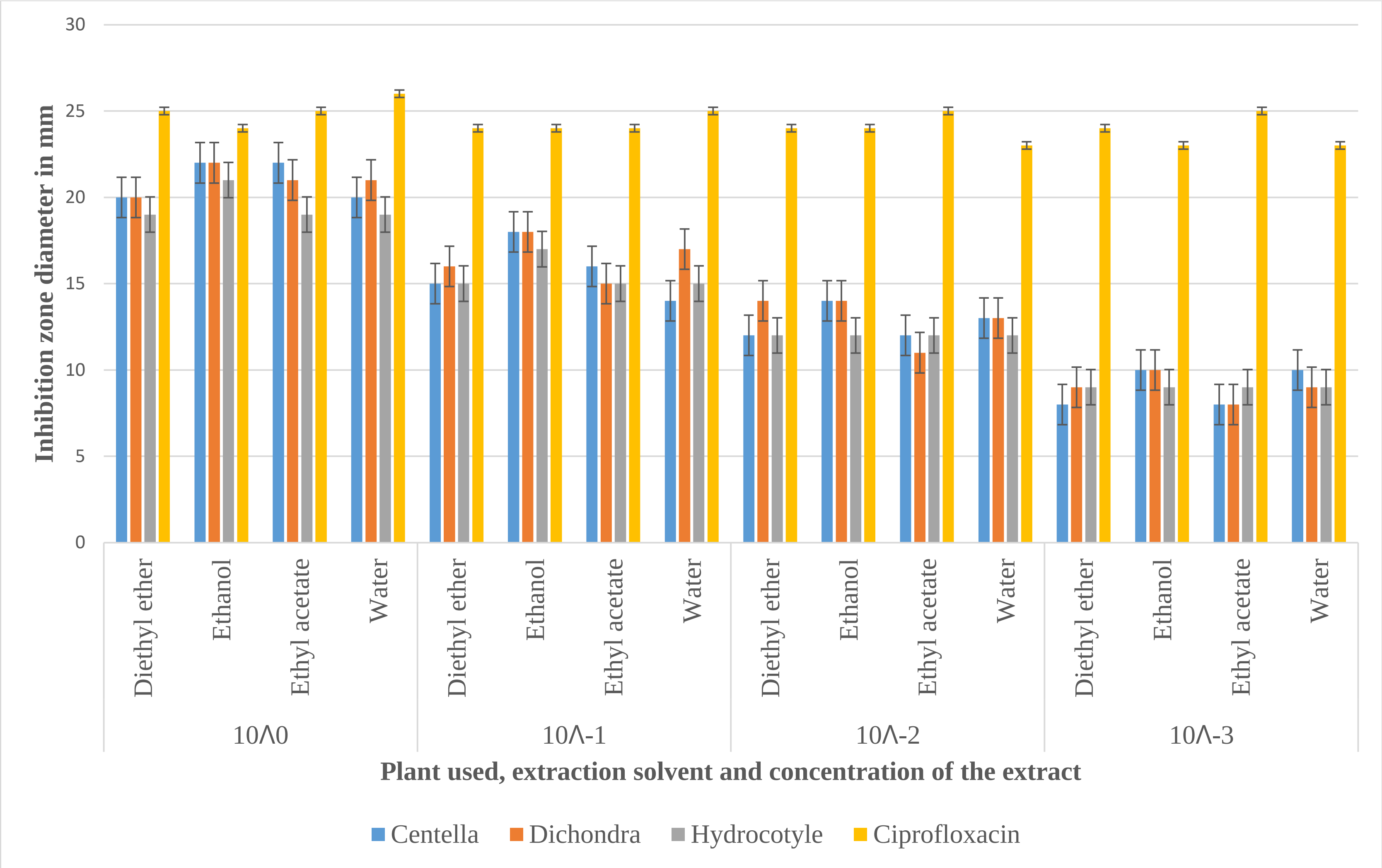

Figure 4.3: Antibacterial activities of *C. asiatica, D. repens* and *H. mannii* against clinical isolate *Pseudomonas aeruginosa*.

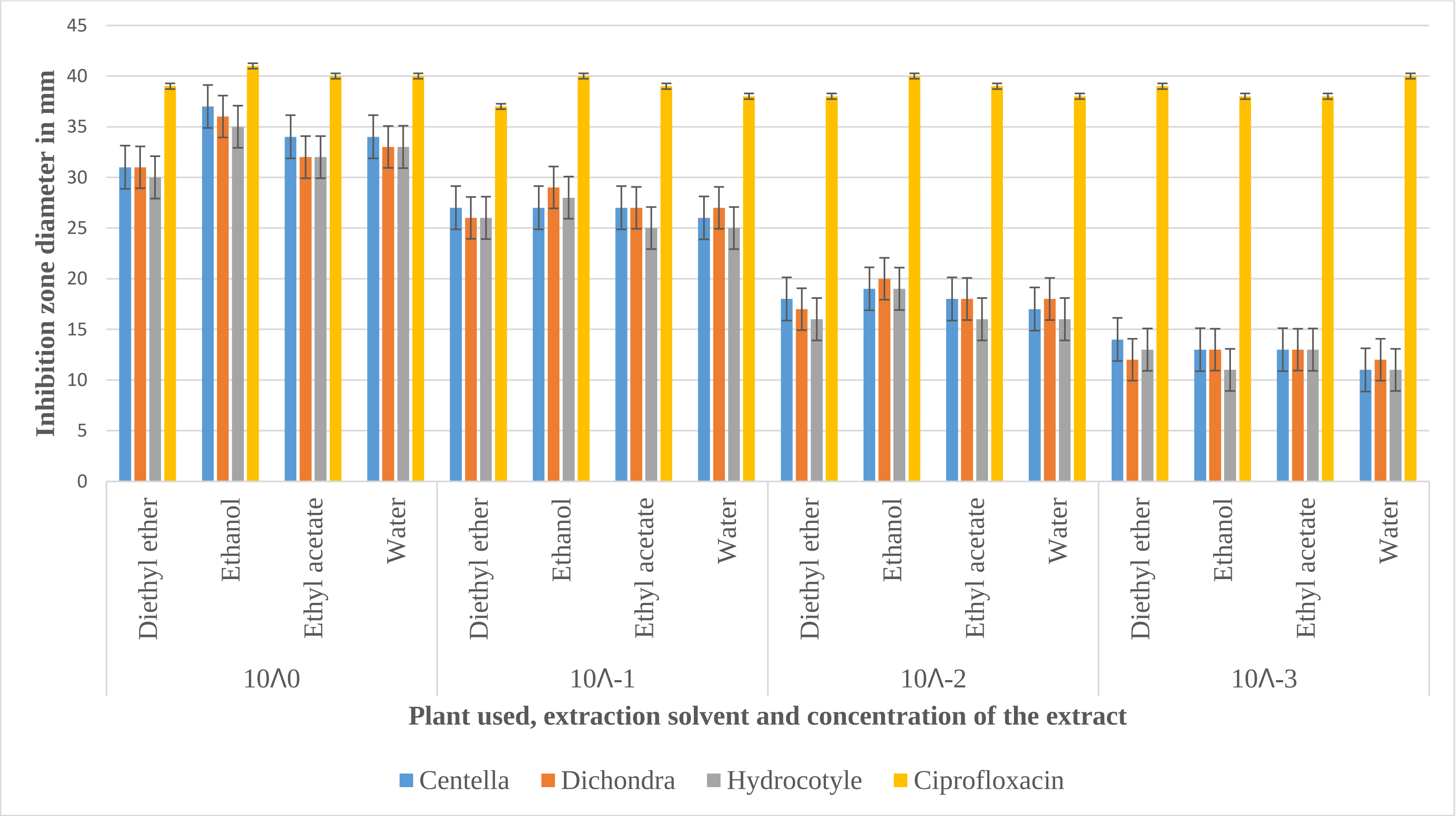

Figure 4.4: Antibacterial activities of *C. asiatica, D. repens* and *H. mannii* against *Staphylococcus aureus* ATCC 25923.

Inhibition zones diameters varied depending on the plant used, concentration of the extract, test microorganism and/or the solvent used. The inhibition zones diameters decreased with decreasing concentration of extracts with 10^{0} having bigger zones compared to 10^{-3} concentrations. Plate 4.1 shows the antibacterial activity of *Centella asiatica* and *Hydrocotyle manii* extracts against *E. coli* and *P. aeruginosa* respectively.

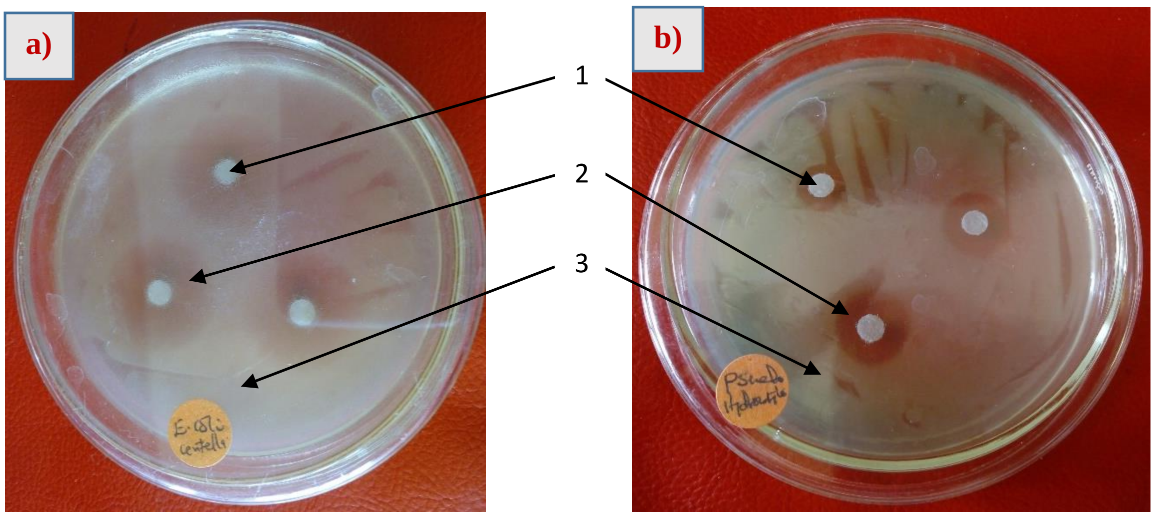

Plate 4.1: *E. coli* on *Centella asiatica* extract (a) and *P. aeruginosa* on *Hydrocotyle manii* extract (b).

Key: **1** – Disc impregnated with extract, **2** – Zone of inhibition, **3** – Bacterial colony

Apart from the main factors (concentration of the extract, solvent used, plant type and the bacterial species), the interactions between concentration x solvent, concentration x plant type, concentration x bacterial species, solvent x plant type, solvent x bacterial species, concentration x solvent x plant type, concentration x solvent x bacterial species, solvent x plant type x bacteria species, as well as concentration x solvent x plant type x bacterial species had a statistically significant effect on the inhibition zone diameters. The interaction between plant type x bacteria species and concentration x plant type x bacteria species were however not significant at 95% confidence level.

Table 4.2 summarizes the relationships between the plant used, concentration of the extract, the test microorganism as well as the solvents used for extraction at $P \leq 0.05$.

Table 4.2: Effects and relationships between plant type, concentration of the extract, the test microorganism and the solvents used for extraction on Inhibition zones diameters

Source of variation	F-Ratio	P-Value	Effect as at p ≤ 0.05
A:Concentration	1534.58	0.0000	Significant
B:Solvent used	30.76	0.0000	Significant
C:Plant type	4.36	0.0137	Significant
D:Bacterial species	481.83	0.0000	Significant
AB	18.18	0.0000	Significant
AC	3.54	0.0021	Significant
AD	33.76	0.0000	Significant
BC	2.69	0.0148	Significant
BD	5.05	0.0001	Significant
CD	0.43	0.7893	Not Significant
ABC	2.71	0.0003	Significant
ABD	2.66	0.0004	Significant
ACD	0.22	0.9975	Not Significant
BCD	2.59	0.0028	Significant
ABCD	1.90	0.0022	Significant

Thirteen P-values were less than 0.05 meaning these factors had a statistically significant effect on inhibition zone diameters (mm) as at the 95.0% confidence level. The p-values ranged from 0.0000 to 0.9975 at $P \leq 0.05$ (Table 4.2). This suggests that the plant extracts being studied can function as antibacterial agents.

4.4 Allelopathic activities of crude extracts

Allelopathic activities of crude extracts on germination was tested on seeds of maize, oats, rice, sorghum and wheat in petri dishes. Aqueous, ethanol, ethyl acetate and diethyl ether extracts of *C. asiatica*, *D, repens* and *H. mannii* were used. Dilutions of extracts was done using double distilled water to make extracts concentrations of 10^0 to 10^{-3}.

4.4.1 Germination percentages.

Surface sterilised seeds of maize, oats, rice, sorghum and wheat were used. There were no seeds that germinated in diethyl ether and ethyl acetate extracts. Oats and wheat did not also germinate in ethanolic extracts. Plates 4.2, 4.3, 4.4, 4.5 and 4.6 below shows the allelopathic effects the plants under investigation on the germination of seeds of the test plants when compared to controls on distilled water.

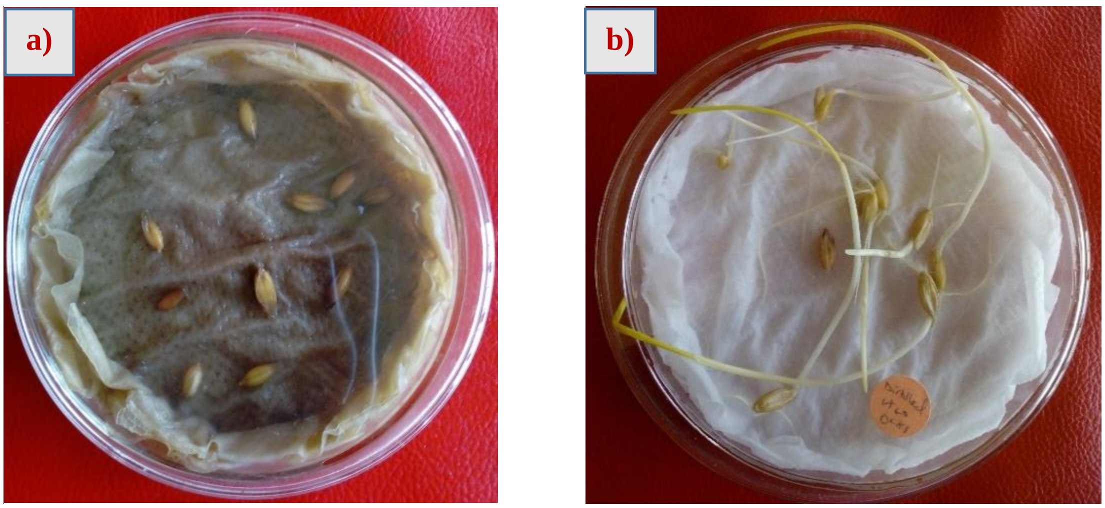

Plate 4.2: Oat seeds on (a) *Dichondra repens* extract and (b) Oat seeds on distilled water

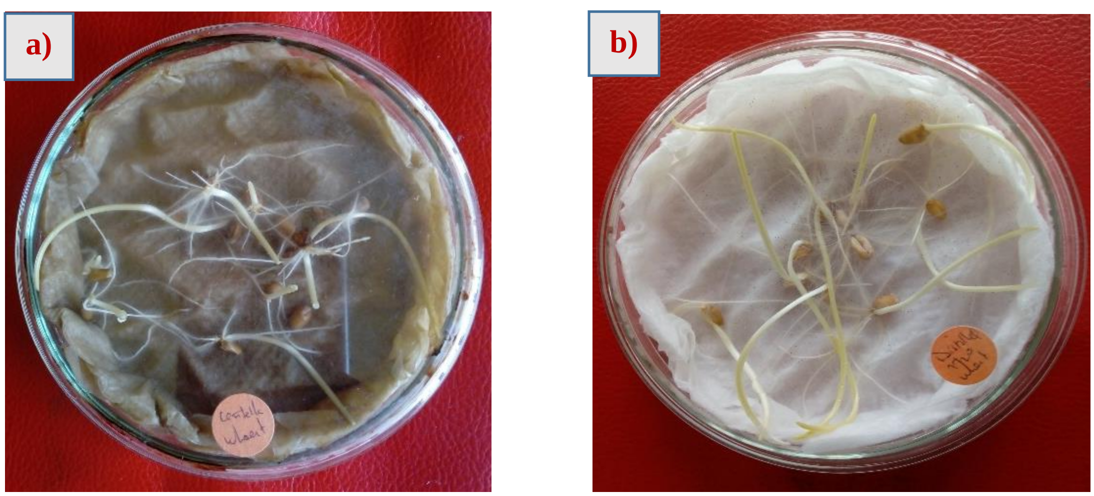

Plate 4.3: Wheat seeds on (a) *Centella asiatica* extract and (b) Wheat seeds on distilled water

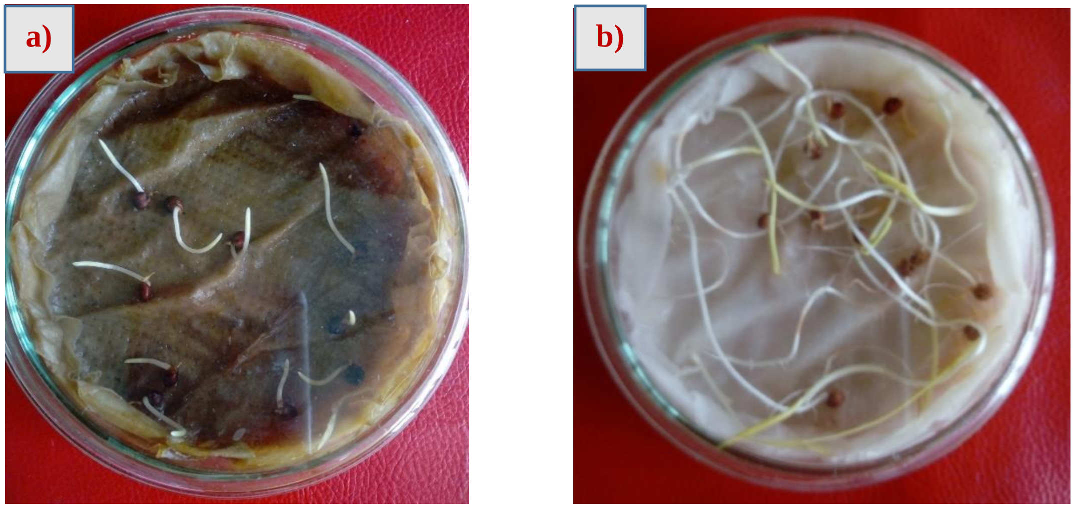

Plate 4.4: Sorghum seeds on *Dichondra repens* extract (a) and Sorghum seeds on distilled water (b).

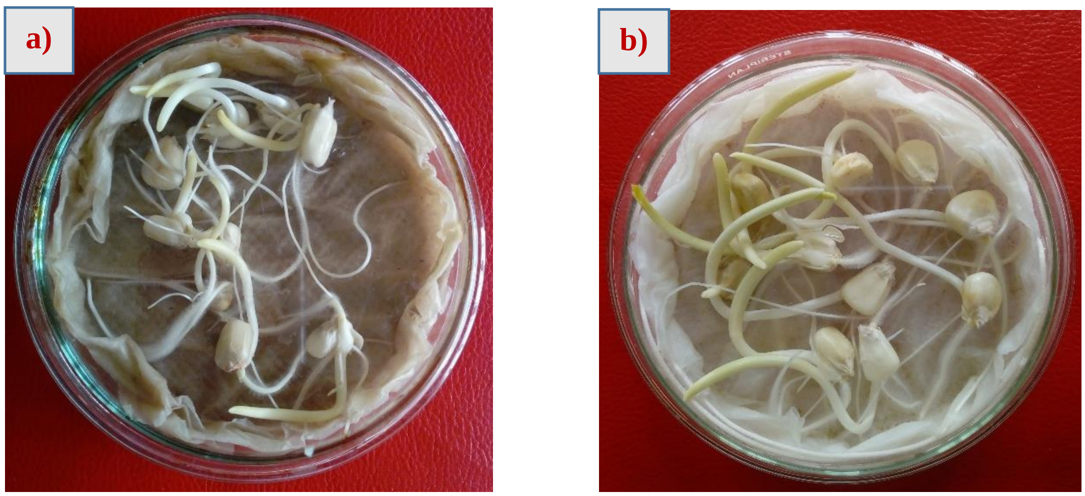

Plate 4.5: Maize seeds on (a) *Centella asiatica* extract and (b) Maize seeds on distilled water

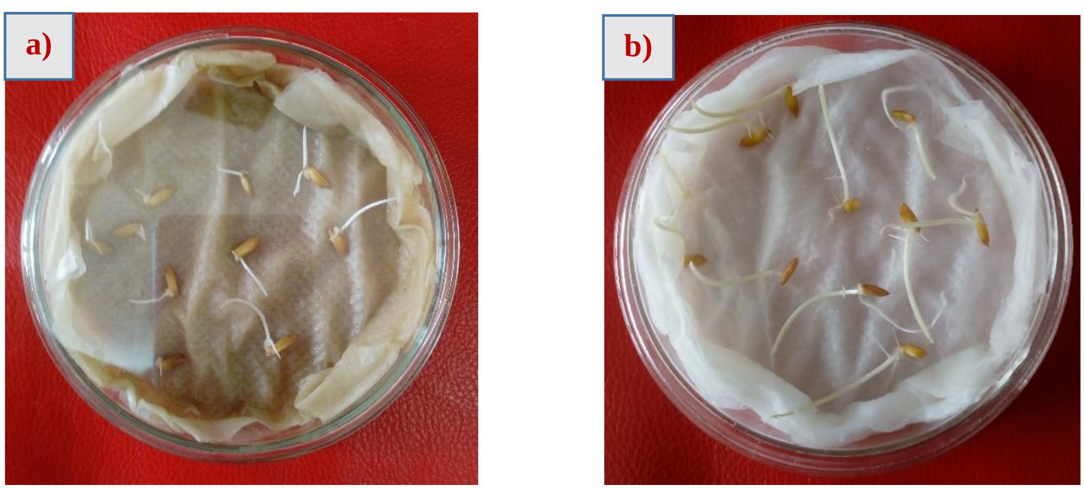

Plate 4.6: Rice seeds on (a) *Hydrocotyle mannii* extract and (b) Rice seeds on distilled water

The number of seeds that germinated was counted after five days (Table 4.3) then used to calculate germination percentages (Table 4.4). Sorghum had the highest germination percentage of 80.36% while oats had the lowest germination percentage of 9.17% respectively. Maize, rice and wheat had germination percentages of 79.76%, 72.44% and 22.5% respectively (Table 4.4).

Table 4.3: Number of seeds that germinated after five days

Solvent	Plant	10^0	10^{-1}	10^{-2}	10^{-3}	+VE	-VE
Water	Maize	177	192	204	198	120	0
	Oats	18	37	32	65	119	0
	Rice	157	182	191	189	120	0
	Sorghum	189	173	190	187	120	0
	Wheat	68	103	95	112	120	0
Ethanol	Maize	109	153	166	141	120	0
	Rice	86	110	144	158	120	0
	Sorghum	132	156	152	171	120	0

Table 4.4: Germination percentage of seeds

Plant	Germinated seeds	Total	Germination %
Maize	1340	1680	79.76
Oats	154	1680	9.17
Rice	1217	1680	72.44
Sorghum	1350	1680	80.36
Wheat	378	1680	22.5

4.4.2 Effects of aqueous and ethanolic plant extracts on plumule length

Based on the original concentration (10^0), the highest percentage reduction in the plumule length was in oats (100%) on *D. repens* aqueous extract with the lowest percentage reduction being in sorghum (40%) on ethanolic extracts of *H. mannii*. The highest percentage reduction in plumule lengths of maize based on the original concentration (10^0) was on ethanolic extracts of *D. repens* (77%) with the lowest percentage reduction being on aqueous extracts of *H. mannii* (23%). However, reducing activity was observed with reducing extract concentrations; from 10^0 to 10^{-3} (Figures 4.5).

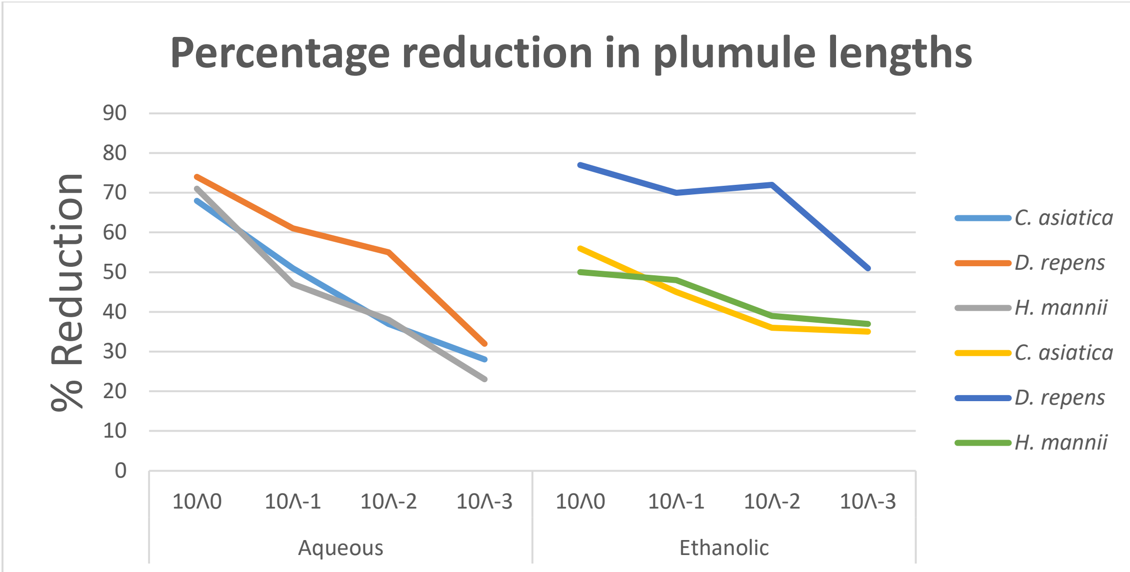

Figure 4.5: Percentage reductions in plumule lengths of maize.

The highest percentage reduction in plumule lengths of rice based on the undiluted concentration (10^0) was on aqueous extracts of *C. asiatica* and *D. repens* with 72% reduction each while the lowest percentage reduction being on ethanolic extracts of *H. mannii* (35%). There was reducing effect with reducing extract concentrations; from 10^0 to 10^{-3} (Figures 4.6).

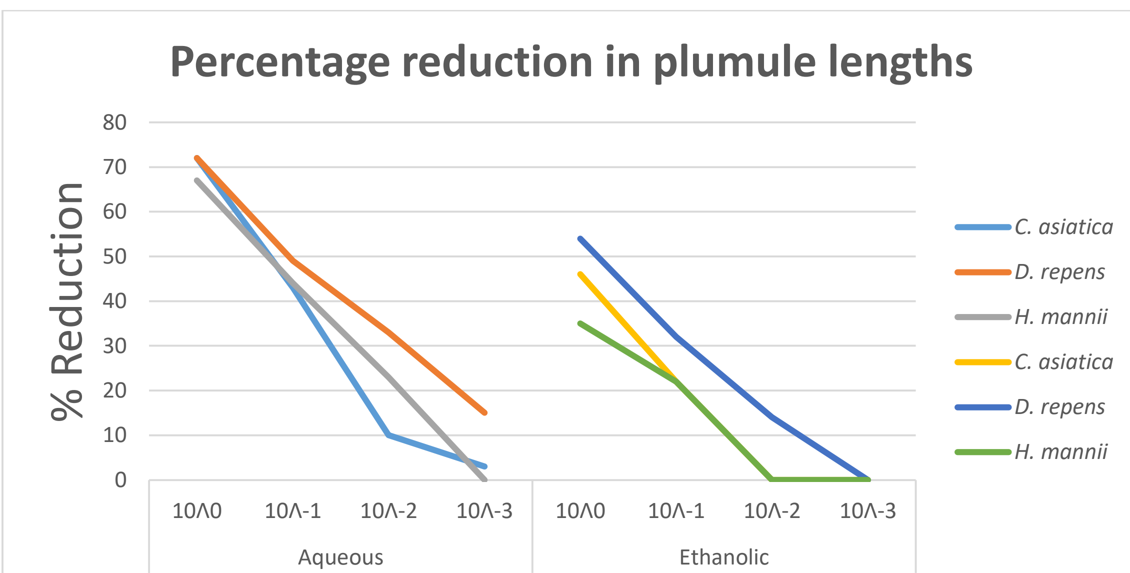

Figure 4.6: Percentage reductions in plumule lengths of rice.

The highest percentage reduction in plumule lengths of sorghum based on the original concentration (10^0) was on aqueous extracts *D. repens* (87%) with the lowest percentage reduction being on ethanolic extracts of *H. mannii* (40%). The activity was decreasing with reducing extract concentrations; from 10^0 to 10^{-3} (Figure 4.7).

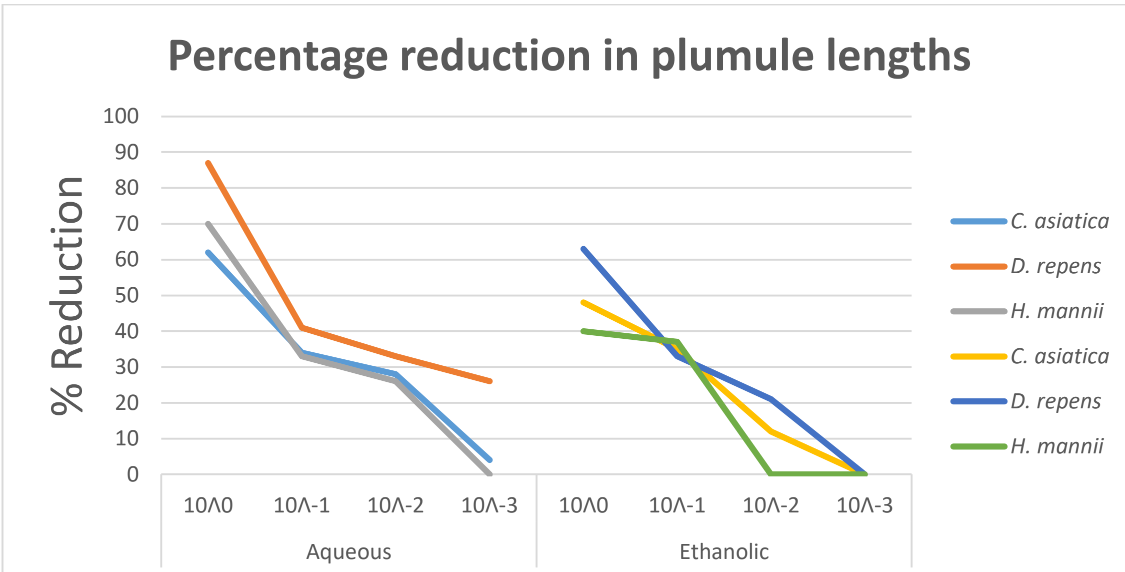

Figure 4.7: Percentage reductions in plumule lengths of sorghum.

Oats and wheat only germinated in aqueous extracts. The highest percentage reduction in plumule lengths of oats based on the original concentration (10^0) was on *D. repens* extracts (100%) with the lowest percentage reduction being on *H. mannii* extracts (87%) (Figure 4.8). percentage reduction based on the original was also on *D. repens* plant extract showed the highest decrease in plumule lengths of wheat (86%) at the concentration 10^0 which was reducing with the decrease in concentrations. However, the effect on the length by *H. mannii* extracts was the lowest (57%) in this case on the concentration 10^0 (Figures 4.8).

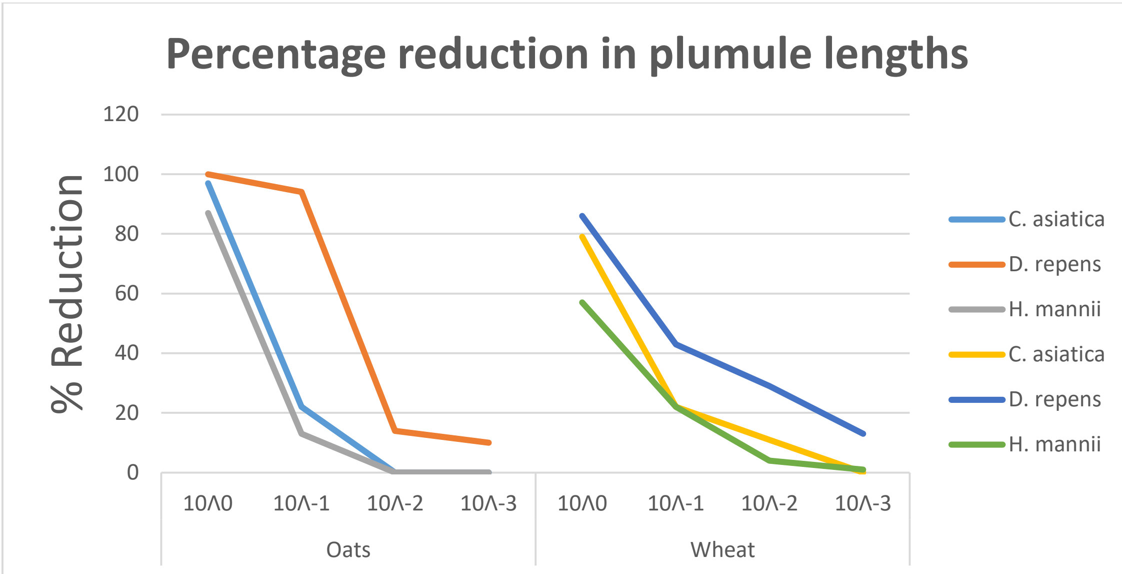

Figure 4.8: Percentage reductions in plumule lengths of oats and wheat.

The main factors (concentration of the extract, solvent used, plant extract used and the plant type under investigation) as well as individual plant extracts (*C. asiatica*, *D. repens* and *H. mannii*) and their interactions (*C. asiatica* x *D. repens*, *C. asiatica* x *H. mannii*, *D. repens* x *H. mannii* and *C. asiatica* x *D. repens* x *H. mannii*) had a statistically significant effect on plumule length at the 95.0% confidence level (Table 4.5).

Table 4.5: Effects and relationships between plant extract used, concentration of the extract, plant type under investigation and the solvents used for extraction on the plumule lengths

Source of variation	F-Ratio	P-Value	Effect as at $p \leq 0.05$
Concentration	1086.29	0.0000	Significant
Solvent used	10.10	0.0015	Significant
Plant extract used	57.68	0.0000	Significant
Plant investigated	1017.98	0.0000	Significant
C:*Centella asiatica*	57.30	0.0000	Significant
D:*Dichondra repens*	53.91	0.0000	Significant
H:*Hydrocotyle mannii*	115.62	0.0000	Significant
C+D	80.46	0.0000	Significant
C+H	147.46	0.0000	Significant
D+H	71.82	0.0000	Significant
C+D+H	88.45	0.0000	Significant

The P-values of the F-tests on individual plant extracts and their interactions were less than 0.05. This indicates that there was a statistically significant difference between the mean plumule lengths from one level of plant to another at the 95.0% confidence level.

The p-values ranged from 0.0000 to 0.0015 (Table 4.5) as at $P \leq 0.05$. Since all P-values were less than 0.05, it means that these factors have a statistically significant effect on plumule length at the 95.0% confidence level. This therefore suggests that the plant extracts under investigation can be utilized to control growth of some plant species in *Gramineae* family.

4.4.3 Effects of aqueous and ethanolic plant extracts on radicle length

Considering the initial concentration (10^0) tested, it showed that highest percentage reduction in the radicle length was in oats (100%) on *Dichondra repens* aqueous extract with the lowest percentage reduction was observed in maize (31%) on ethanolic extracts of *Hydrocotyle mannii* (Figure 4.9).

The highest percentage reduction in radicle lengths of maize based on the original concentration (10^0) was on ethanolic extracts *D. repens* (52%) with the lowest percentage reduction being on aqueous extracts of *H. mannii* (24%) which exhibited a reducing activity with reducing extract concentrations; from 10^0 to 10^{-3} (Figure 4.9).

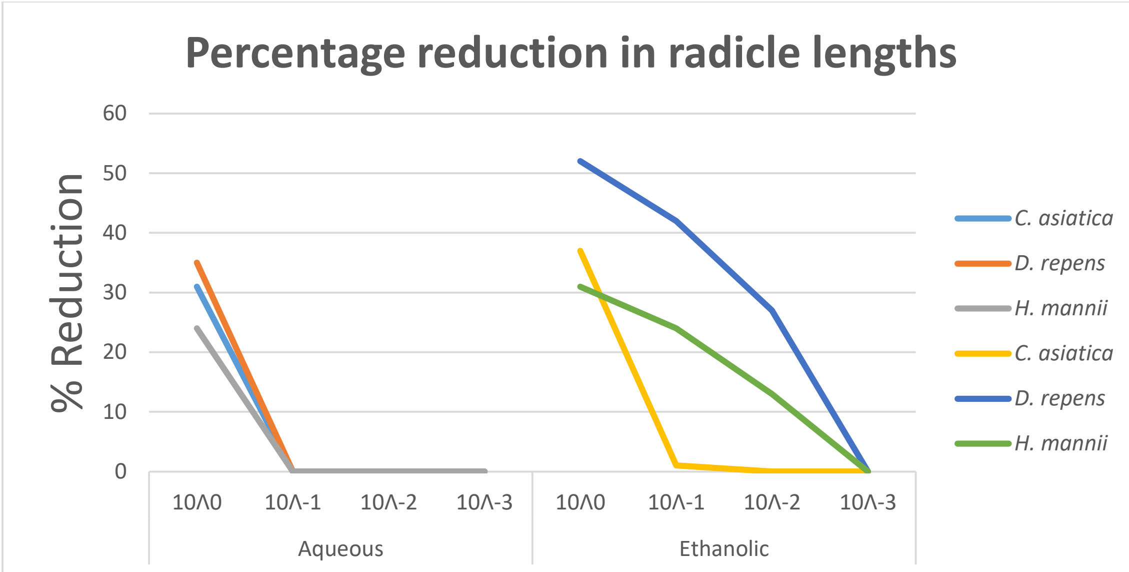

Figure 4.9: Percentage reductions in radicle lengths of maize.

The highest percentage reduction in radicle lengths of rice was on ethanolic extracts of *D. repens* (76%) at concentration 10^0 with the lowest percentage reduction being on aqueous extracts of *H. mannii* (39%). There was a reduction in the effect of the plant extracts as the concentration decreases (Figure 4.10).

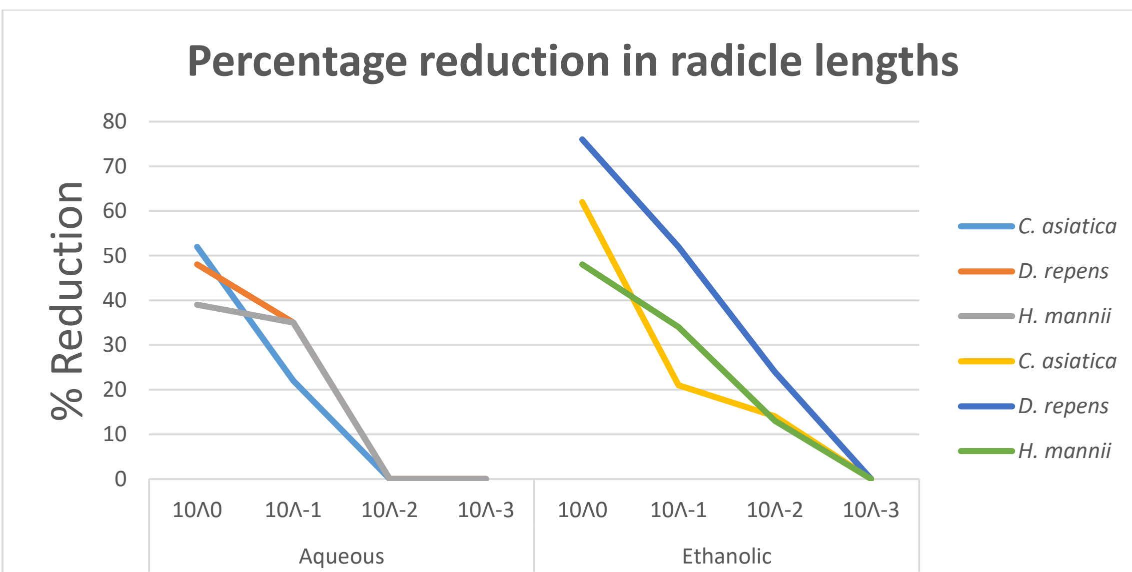

Figure 4.10: Percentage reductions in radicle lengths of rice.

The highest percentage reduction in radicle lengths of sorghum based on the original concentration (10^0) was on aqueous extracts *D. repens* (97%) with the lowest percentage reduction being on ethanolic extracts of *H. mannii* (56%). There was reducing activity with reducing extract concentrations; from 10^0 to 10^{-3} (Figure 4.11).

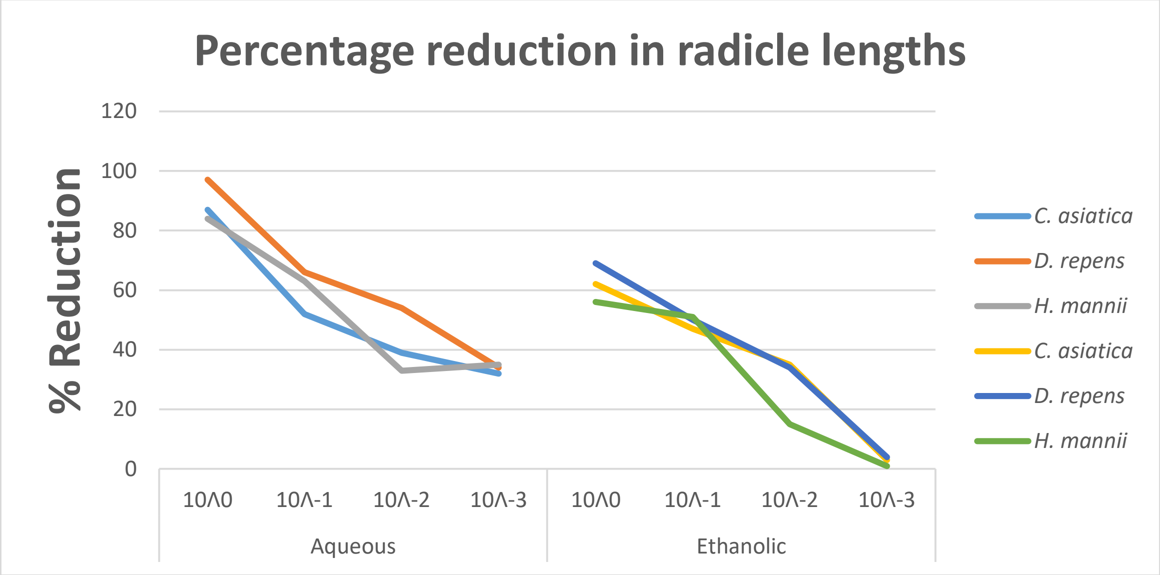

Figure 4.11: Percentage reductions in radicle lengths of sorghum.

Oats and wheat only germinated in aqueous extracts. The highest percentage reduction in radicle lengths of oats at the concentration 10^0 was on *D. repens* extracts (100%) with the lowest percentage reduction being on *C. asiatica* extracts (96%) (Figure 4.12). A reduction in radicle lengths of 94% by the effect of *D. repens* was noted in wheat seedlings. However, *H. mannii* extracts could only reduce the lengths by 76%. In all the effect, it was observed that the lengths increased with a decrease in concentration (Figure 4.12).

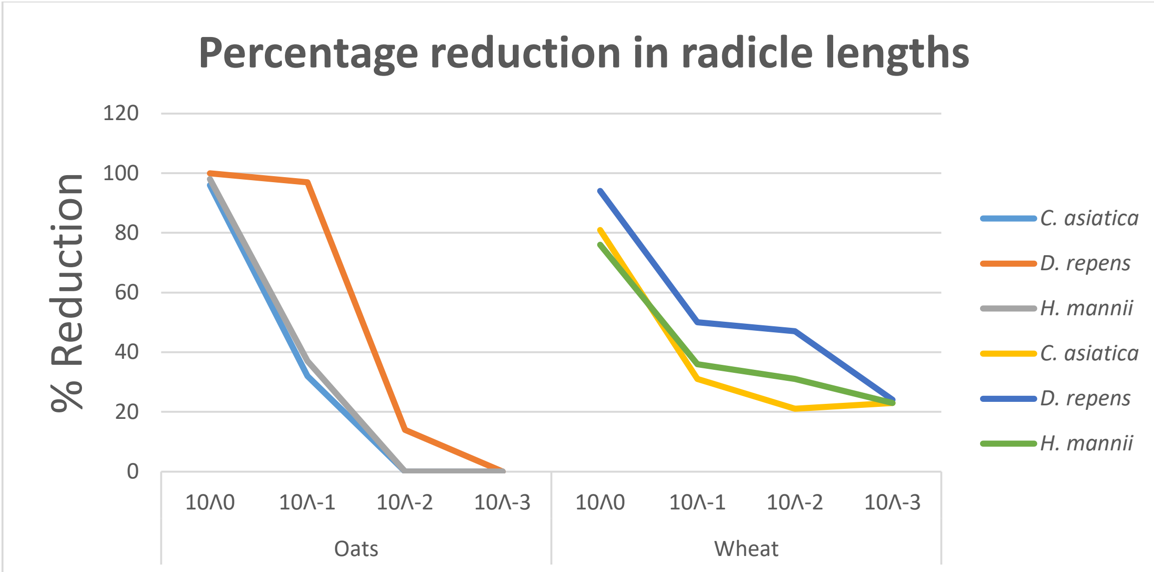

Figure 4.12: Percentage reductions in radicle lengths of oats and wheat.

MANOVA was used to determine the effects of the plants extracts on the radicle lengths of the test plants. Table 4.6 below summarizes the effects and relationships between plant extract used, concentration of the extract, plant type under investigation and the solvents used for extraction on radicle lengths at $P \leq 0.05$, which was found to be significantly different in all cases.

Table 4.6: Effects and relationships between plant extract used, extracts concentration, plant type under investigation and the solvents used for extraction on radicle lengths

Source of variation	F-Ratio	P-Value	Effect as at $p \leq 0.05$
Concentration	960.25	0.0000	Significant
Solvent used	12.89	0.0003	Significant
Plant extract used	25.75	0.0000	Significant
Plant investigated	2692.19	0.0000	Significant
C:*Centella asiatica*	266.46	0.0000	Significant
D:*Dichondra repens*	162.19	0.0000	Significant
H:*Hydrocotyle mannii*	235.11	0.0000	Significant
C+D	199.98	0.0000	Significant
C+H	313.83	0.0000	Significant
D+H	178.52	0.0000	Significant
C+D+H	211.40	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 (*C. asiatica, D. repens* and *H. mannii*) and their interactions (*C. asiatica* x *D repens*, *C. asiatica* x *H. mannii*, *D. repens* x *H. mannii* and *C. asiatica* x *D. repens* x *H. mannii*) had a significant effect on radicle length (Table 4.6). Just as it was in the case of plumule lengths, the P-values of the F-tests on individual plant extracts and their interactions were also less than 0.05. This indicates that there is a statistically significant difference between the mean radicle lengths from one level of plant to another at the 95.0% confidence level.

The p-values ranged from 0.0000 to 0.0003 at $P \leq 0.05$ (Table 4.6). Since all P-values are less than 0.05, it means that these factors have a statistically significant effect on radicle length at the 95.0% confidence level. This therefore also implies that the plant extracts under investigation can be used to control growth of plant species in *Gramineae* family.

4.5 Phytochemicals present

Qualitative phytochemical screening was conducted on the plant extracts for alkaloids, coumarins, flavonoids, glycosides, phenols, quinones, saponins, steroids, tannins and terpenoids. Out of the ten phytochemicals screened, only glycosides were absent in any of the extracts of the three test plants. Aqueous plant extracts possessed more than half of the screened phytochemicals unlike diethyl ether extracts. Diethyl ether extracted coumarins, phenols, steroids and tannins. Ethyl acetate extracted alkaloids, coumarins, phenols and quinones. Ethanol extracted alkaloids, coumarins, phenols, quinones, saponins and tannins. Water extracted coumarins, flavonoids, quinones, saponins, steroids, tannins and terpenoids (Table 4.7). Plates 4.7, 4.8 and 4.9 shows some of the phytochemicals qualitatively screened for their presence or absence in the plant extracts.

Table 4.7: Qualitative phytochemicals on tested plant type extracted from different solvents.

Plant extract	**Solvent Used**	**Phytochemicals**									
		Alkaloids	Coumarins	Flavonoids	Glycosides	Phenols	Quinones	Saponins	Steroids	Tannins	Terpenoids
C. asiatica	Diethyl ether	-	-	-	-	+	-	-	+	-	-
D. repens	Diethyl ether	-	+	-	-	+	-	-	-	+	-
H. mannii	Diethyl ether	-	-	-	-	+	-	-	-	+	-
C. asiatica	Ethanol	+	-	-	-	+	+	+	-	-	-
D. repens	Ethanol	+	+	-	-	-	-	+	-	-	-
H. mannii	Ethanol	+	-	-	-	-	-	+	-	+	-
C. asiatica	Ethyl acetate	+	-	-	-	+	+	-	-	-	-
D. repens	Ethyl acetate	+	+	-	-	+	-	-	-	-	-
H. mannii	Ethyl acetate	+	-	-	-	-	-	-	-	-	-
C. asiatica	Water	-	+	+	-	-	+	+	+	+	+
D. repens	Water	-	+	+	-	-	+	+	+	+	+
H. mannii	Water	-	+	+	-	-	+	+	+	+	+

Key:

+ = Present

- = Absent

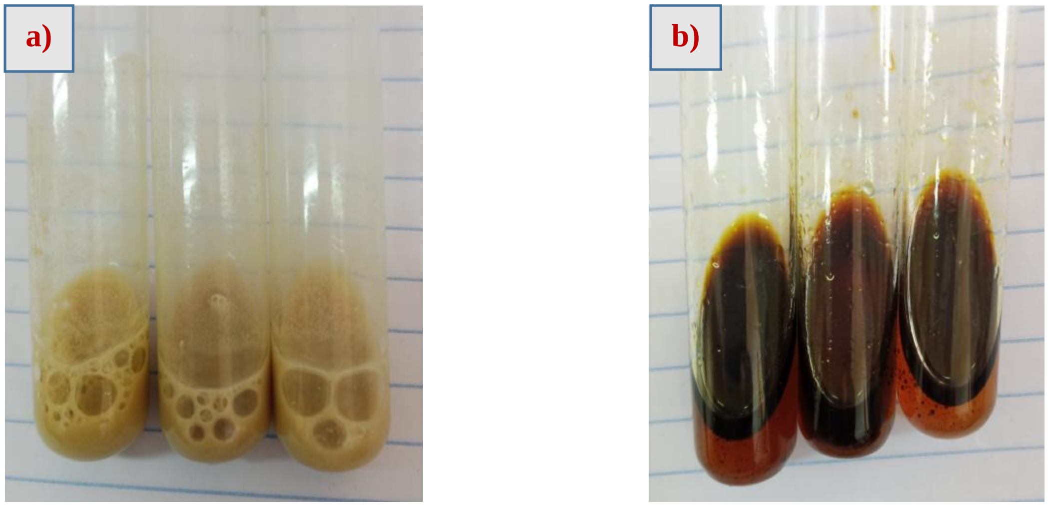

Plate 4.7: Presence of Flavonoids (a) and presence of Terpenoids (b) in the extracts.

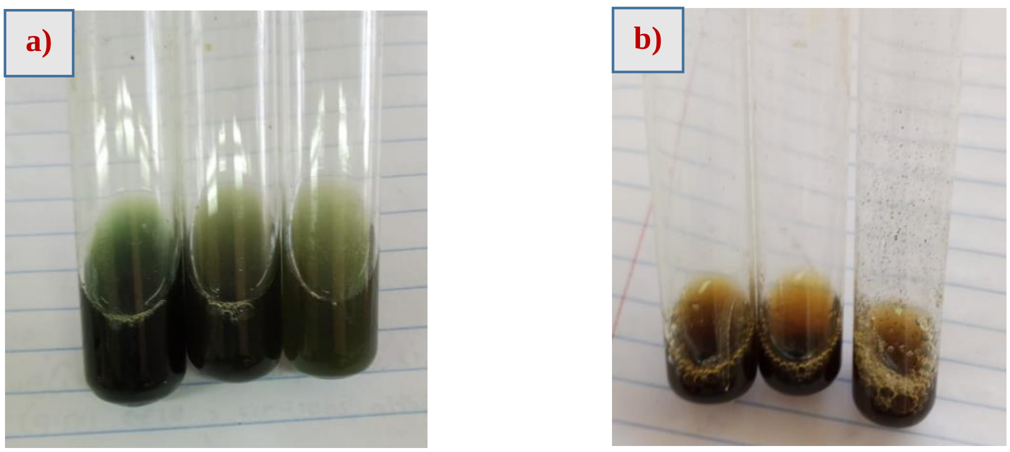

Plate 4.8: Presence of Tannins (a) and presence of Alkaloids (b) in the extracts.

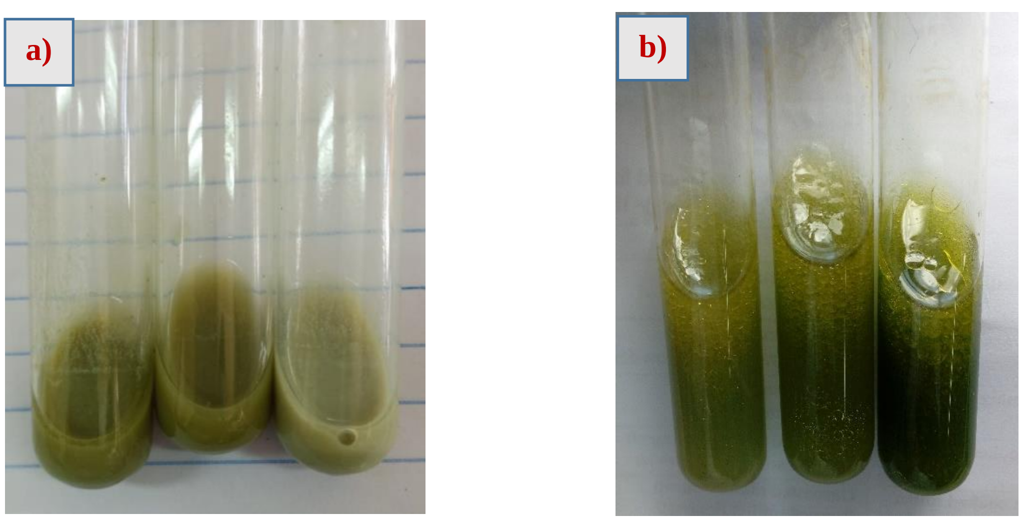

Plate 4.9: Presence of Coumarins (a) and presence of Saponins (b) in the extracts.

CHAPTER FIVE

DISCUSSIONS

5.1 Percentage yields of plant samples

The mean percentage yield of the plant leaves was higher in water and ethanol when compared to ethyl acetate and diethyl ether. This indicates that extraction efficiency favours solvents with highly polarity (Abubakar *et al.,* 2017; Truong *et al.,* 2021). The mean percentage yield of the three plants didn't differ greatly on average. This can be attributed to their existence in the *Hydrocotylo-Centelletum asiaticae* association which is dominated by *C. asiatica* and *H. mannii* (Mosango, 2017).

Crude extracts of *C. asiatica*, *D. repens* and *H. mannii* were prepared from their leaves as crude extracts which are known to possess both active and non-active substances. These crude extracts have also been documented to confer higher efficacy as compared to pure or semi-crude plant extracts (Agrawal *et al.,* 2012).

5.2 Efficiency of solvents used for extraction

The mean percentage yields also varied depending on the solvents used in the extraction process. Solvent used is considered one of the most important factor influencing efficiency of extraction (Truong *et al.,* 2021). In this study, water produced the highest mean crude extract percentage, followed by ethanol and ethyl acetate with diethyl ether having the least mean percentage yield. This phenomenon can be attributed to the polarity index of solvents used for extraction. Water has higher polarity index of 10.2, ethyl acetate is 4.4, ethanol is 4.3 and diethyl ether is 2.8. 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 of this study.

5.3 Antibacterial activities of crude extracts

The results from this study showed that the aqueous, diethyl ether, ethanol and ethyl acetate extracts of C. *asiatica*, *D. repens* and *H. mannii* have antibacterial activities against *Escherichia coli* ATCC 25922, clinical isolate of *Pseudomonas aeruginosa* and *Staphylococcus aureus* ATCC 25923 supporting Doctrine of Signatures theory on the discovery and utilisation of medicinal plants (Bennett *et al.*, 2007).

Extracts of *C. asiatica*, *D. repens* and *H. mannii* on average had a similar antibacterial effect on the test microorganism. This demonstrated that none of the plant extracts was more potent than the others justifying why the three plants can be found growing together and are commonly termed the *Hydrocotylo-Centelletum asiaticae* association (Mosango, 2017).

The plants might have had similar effect when compared to each other but their effect varied on the test organisms. Among test pathogens, the extracts had greater antibacterial activity on *S. aureus* followed by *E. coli* with little activity on *P. aeruginosa*.

Medicinal plants for quite some time have been documented to have therapeutic activities (Prakash *et al.*, 2017; Zahara *et al.*, 2021). It is therefore of no surprise given the antibacterial activity of *C. asiatica*, *D. repens* and *H. mannii* as justified in this study. These findings concur with prior documented studies by other authors. Several plant extracts have been reported to be active on *E. coli*, *P. aeruginosa* and *S. aureus* with *C. asiatica* which is a member of the *Hydrocotylo-Centelletum asiaticae* association being documented by authors like Prakash *et al.* (2017) and Zahara *et al.* (2021). Prakash *et al.* (2017) documented the antibacterial activity of methanolic extracts of *C. asiatica* leaves on both gram-positive *S. aureus* ATCC 25923 and methicillin resistant *S. aureus*. Zahara *et al.* (2021) reported that diethyl ether, dichloromethane, ethyl acetate, methanol and hexane extracts of *C. asiatica* leaves inhibited *K. aerogenes*, *P. vulgaris*, *B. subtilis* and *S. aureus*. Methanol extracts of *C. asiatica* leaves have also been documented to inhibit *B. cereus*, *E. coli*, *S. aureus* and *P. aeruginosa* (Arumugam *et al.,* 2011). This study findings contribute to the knowledge on the effectiveness of the test plants extracts against *S. aureus*, *E. coli* and *P. aeruginosa*.

The solvent that was employed during the extraction process had a significant impact on the antibacterial activity of each extract. Different levels of antibacterial activity were present in extracts with ethanol-based extracts showing a higher activity compared to aqueous, diethyl ether and ethyl acetate extracts. This can be attributed to alcoholic aqueous environment created by ethanol as a solvent which facilitates ease of extraction as well as high saponins, alkaloids and antioxidants (Bukar *et al.,* 2013).

5.4 Allelopathic activities of crude extracts

Allelopathic activities of crude extracts on germination was tested on seeds of maize, oats, rice, sorghum and wheat which are members of *Gramineae* family. Oats and wheat did not also germinate in ethanolic extracts of the test plant extracts. This means that those extracts were detrimental to the growth and germination of these plants. *D. repens* extracts were more detrimental to the germination and growth of the test plant species as evident in the percentage reductions when compared to *C. asiatica* and *H. mannii* extracts. This could indicate a higher degree of allelopathy of *D. repens* as initial colonizers of virgin lands and their dominance in grassland habitats of forests in Kenya as reported by Wanjohi *et al.* (2017).

The findings of this study showed that *C. asiatica*, *D. repens* and *H. mannii* leaf extracts decreased germination percentages, radical length and plumule lengths of the test field crops as the extract concentration increased which concurs with Raoof & Siddiqui, (2012). All the p-values were less than 0.05 at 95.0% confidence level which clearly indicate that the plumule and radicle lengths were affected significantly. This therefore implies that *C. asiatica*, *D. repens* and *H. mannii* leaf extracts can be used to control growth of the test plants used in the study. This is in line with prior research which have documented before particularly on *C. asiatica* which has been better studied when compared to *D. repens* and *H. mannii* which have limited literature.

When the effects of *C. asiatica* stem and leaf extracts were examined on both fresh and dry weight in rice, Alagesaboopathi, (2018) discovered that the lengths of the plumule and radicle were greatly shortened. Similar results for pearl millet and cow pea using aqueous extracts of *C. asiatica* were obtained (Alagesaboopathi, 2010).

Since *C. asiatica, D. repens* and *H. mannii* grows as weeds, results of this study can also be compared to studies on other weeds. The effects of *Argemone mexicana* L. water extract on *Sorghum bicolor* (L.) Moench seed germination and seedling growth were examined by Alagesaboopathi (2013). The author reported a reduction of 30% and 49% in plumule and radicle lengths respectively. Majeed *et al.* (2012) and Mandal *et al.* (2016) tested the effects of weeds extracts on wheat. Aqueous extracts of *Chenopodium album* L. significantly suppressed the plant height of wheat corresponding to lower grain yield (Majeed *et al.*, 2012). According to Mandal *et al.* (2016), aqueous leaf extracts of *Andrographis paniculata* reduced the length of wheat shoots and roots as well as its dry weight. Anwar *et al.* (2019) reported leaf extracts of *Carica papaya* to be allelopathic against the growth and development of major weeds of wheat. The extracts inhibited seed germination of *Euphorbia helioscopia* (50%), *Phalaris minor* (45%) and *Avena fatua* (41%). Alagesaboopathi & Thamilazhagan, 2010, reported that leaves and stem extracts of *Andrographis lineata* decreased significantly seedling growth and germination percentages of green gram and balckgram. Similar results were reported using *Andrographis paniculata* on *Sesamum indicum* L. (Alagesaboopathi, 2011).

Although not established in the current study, previous studies have alleged that extracts from weeds may possess some plant growth inhibitors and phototoxic chemicals which are involved in allelopathy (Raoof & Siddiqui, 2012). They include growth regulators, some nutrients, phytochemicals and toxins (Mandal *et al.*, 2016; Alagesaboopathi, 2018). When compared to other cultivars in both lab and greenhouse experiments, the Japanese cultivar lucerne was found to be extremely inhibitive of seedling germination and growth linked to large flavonoid concentrations. However, their role in allelopathic interference has not been well characterized (Weston & Mathesius, 2013). The observed decrease in seed germination, especially on oats, may potentially be the result of the seeds' restriction of water absorption.

The findings of this study have put to light allelopathic activities of *C. asiatica*, *D. repens* and *H. mannii*. Therefore, there is a high likelihood for poor growth performance of maize, oats, rice, sorghum and wheat which are major staple foods in the presence of *C. asiatica*, *D. repens* and *H. mannii* in the fields which could have an impact in reducing yields, financial losses to farmers and eventually food insecurity.

5.5 Phytochemicals present

Results showed that all the solvents used for extraction were able to extract some phytochemicals which varied from one solvent to another. In this study, alkaloids, coumarins, flavonoids, glycosides, phenols, quinones, saponins, steroids, tannins and terpenoids were detected. However, it is only glycosides that were not detected in any extracts of *C. asiatica*, *D. repens* or *H. mannii*. Water extracted more phytochemicals when compared to the other solvents used for extraction. Water has a polarity index of 10.2 when compared to ethyl acetate (4.4), ethanol (4.3) and diethyl ether (2.8). The difference in the polarity of the solvents has been documented to affects the solubility of the constituents in each solvent (Abubakar *et al.*, 2017). This explains why water extracts possessed more than half of the screened phytochemicals unlike diethyl ether extracts. The chemical nature of each phytochemical(s) present in plant(s) varies, which then influences greatly their solubility in any given solvent (Abubakar *et al.*, 2017). This can explain the absence of glycosides in any of the extracts of the three test plants. It is also possible that some of the active antimicrobial substances may not have been extracted or some of the volatile compounds may have been lost during the heating of the plant material to evaporate the solvents used for the extraction.

Phytochemicals of plants have been documented to have a wide variety of biological activities including antibacterial and allelopathic activities. Sunday *et al.*, (2012) reported that phytochemical compounds responsible for allelopathic properties also possessed by medicinal plants. This is also evident from the findings of this study, where extracts of *C. asiatica*, *D. repens* and *H. mannii* have exhibited both antibacterial and allelopathic activities. Alkaloids have been documented to be toxic against cells of foreign organisms

(Wintola & Afolayan, 2015). Tannins work against bacteria by depriving them of iron, forming hydrogen bonds, or engaging in specific interactions with their enzymes (Njume *et al.,* 2009). Saponins have also been reported to produce inhibitory effects which affect microbial cells (Wintola & Afolayan, 2015).

According to Abu-Romman *et al.,* (2010), effects of allelochemicals are not only limited to germination inhibition alone but they also result in reduction in the lengths of the targeted plants' radicles and plumules due to disturbance of their regular metabolic processes. The current study findings documents reduced germination percentages as well as plumule and radicle lengths of maize, rice, sorghum, oats and wheat in extracts of *C. asiatica*, *D. repens* and *H. mannii*. The presence of phytochemicals can therefore be linked to these observations. Phenols prevent seeds from germinating by interfering with the respiratory enzymes' functions (Muscolo *et al.,* 2001). Flavonoids have been shown to interact with fundamental molecular targets by inhibiting DNA polymerase I, reverse transcriptase, and protein production, which results in membrane leakage. They have also been found to impair the ATPase function of plasma membranes. (Sunday *et al.,* 2012).

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS.

6.1 Conclusions

The mean percentage yield achieved after extraction in this study was from 14.65% for *Dichondra repens*, 14.21% for *Centella asiatica* and 13.88% for *Hydrocotyle mannii*. Water crude extract had the highest mean percentage yield of 15.87% while diethyl ether had the least mean of 13.2%. Ethanol and ethyl acetate had 14.23% and 13.68% respectively. The aqueous, ethanol, ethyl acetate and diethyl ether extracts of *C. asiatica*, *D. repens* and *H. mannii* showed significant inhibitory effect against tested bacteria. The plants might have had similar effect when compared to each other. However, their effect varied on the test bacteria exhibiting better activity on *S. aureus* followed by *E. coli* and little activity on *P. aeruginosa*. This therefore implies that the investigated plant extracts can be used as antibacterial agents.

On the test plants extracts, sorghum had the highest germination percentage of 80.36% while oats had the lowest germination percentage of 9.17%. There was however no germination in diethyl ether and ethyl acetate extracts. Oats and wheat did not also germinate in ethanolic extracts. Oats had the highest percentage reduction with the lowest percentage reduction being in sorghum. The extracts of *C. asiatica*, *D. repens* and *H. mannii* showed significant inhibitory effect on seed germination as well as plumule and radicle lengths of the test field crops. This therefore implies that the investigated plant extracts can be used to control growth of other plant species in *Gramineae* family.

Phytochemicals have been documented by various authors as responsible for antimicrobial and allelopathic activities of plants. In this study, alkaloids, coumarins, flavonoids, phenols, quinones, saponins, steroids, tannins and terpenoids were present in the extracts. Since phytochemical compounds possess both antibacterial and allelopathic properties, this study concludes that the phytochemicals present in the extracts of the plants under investigation were responsible for the antibacterial and allelopathic activities of the plants to varying degrees.

6.2 Recommendations

The current study's findings are important but more can be done including the following;

1. The study only determined the antibacterial activities and allelopathic effects of test plants. Their cytotoxicity and the mode of action can also be undertaken.
2. Since crude extracts were used in the study, further purification of extracts can be undertaken to produce semi-crude or pure plant extracts.
3. The current study utilized individual plant extracts to assess their activity. It therefore recommends the use of a combination of the plant extracts to assess for possible synergies.
4. The study only focused on allelopathic effects on seed germination. Seedling growth, crop productivity and yields can also be assessed.
5. Maize H6213 variety, oats S18 variety, rice NERICA L-19 variety, sorghum E-1291 variety and wheat durum variety were used to assess for allelopathic potential of *C. asiatica*, *D. repens* and *H. mannii*. This study recommends the use of other varieties of each test plants as well.
6. There is also need for sensitization for the use of medicinal plants in the management of otitis media associated ailments caused by common pathogens particularly by *E. coli*, *P. aeruginosa* and *S. aureus*.

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APPENDICES

APPENDIX I: Preparation and extraction of crude extracts

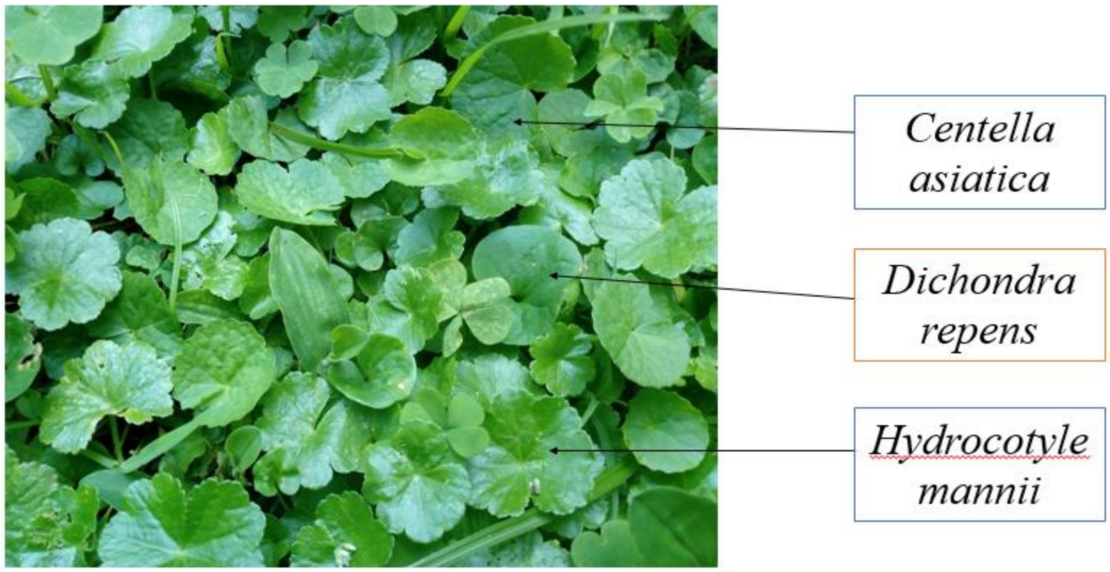

Photo: Kemboi – 2019/10/19 Latt 0.1443 Long 35.1554 Alt 1927.6

a. Plants of interest as they exist in the field

b. Plants parts picked from the field

c. Ground plant leaves

d. Agitation of plant extracts in a shaker.

e. Concentration of extracts using a rotary evaporator

f. Pastes resulting from concentration of extracts

APPENDIX II: Letter of acceptance for publication

AFRICA ENVIRONMENTAL REVIEW Journal

Journal of the School of Environmental Studies,
University of Eldoret,
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Eldoret, Kenya.
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ISSN: 1727 - 8341

30th August, 2022

Dear Kemboi Moses, Pascaline Jeruto , Lizzy Mwamburi and Richard Korir,

RE: LETTER OF ACCEPTANCE

It's my pleasure to inform you that, after the peer review, your paper entitled, **'Allelopathic Potential of *Centella asiatica* Leaves on Seed Germination and Seedling growth of Selected Field Crops'** has been ACCEPTED for publication in the Environmental Review Journal **ISSN: 1727-8341**. The article will be published in **September, 2022 edition** of the Journal Volume 5 No. 2.

Warm regards.

Yours faithfully,

Prof. E. K. Ucakuwun
Editor-in-Chief, AER Journal

AER 5-2-17

AFRICA ENVIRONMENTAL REVIEW Journal

APPENDIX III: Certificate of participation at the 2nd postgraduate conference

P-26

University of Eldoret
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University of Eldoret is ISO 9001:2015 Certified

Certificate
OF PARTICIPATION

This is to certify that

Kemboi Moses, Lizzy Mwamburi, Pascaline Jeruto and Richard Korir

Presented a Topic Titled:

Antibacterial Activities of *Centella asiatica, Dichondra repens* **and** *Hydrocotyle mannii*

During the 2nd Postgraduate Students Conference (Virtual) with the theme:
LEVERAGING ON RESEARCH AND INNOVATION FOR SUSTAINABLE DEVELOPMENT
held on 19 - 20 May, 2022 at University of Eldoret

PROF. PHILLIP RABURU
Deputy Vice-Chancellor
(Planning Reserach & Extension)

PROF. VINCENT SUDOI
Director, Research & Innovation

APPENDIX IV: Similarity report

Turnitin Originality Report

ANTIBACTERIAL ACTIVITIES, ALLELOPATHICEFFECTS AND PHYTOCHEMICAL COMPOSITION OF Centella asiatica, Dichondra repens ANDHydrocotylemannii by Moses Kemboi

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