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BIOPROSPECTING FOR EFFECTIVE ANTIBIOTICS FROM SELECTED KENYAN MEDICINAL PLANTS AGAINST FOUR CLINICAL *Salmonella* ISOLATES

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Bioprospecting for Effective Antibiotics from Selected Kenyan Medicinal Plants against four Clinical *Salmonella* Isolates

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A thesis submitted in fulfillment for the Degree of Doctor of Philosophy in Biochemistry in the Jomo Kenyatta University of Agriculture and Technology

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

This thesis is dedicated to my beloved wife and children for their support and prayers.

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ABBREVIATIONS AND ACRONYMS

ADR	Adverse Drug Reactions
amu	atomic mass units
ANOVA	Analysis of variance
BALB	Baggy Albino (Inbred research mouse)
BRI	Biotechnology Research Institute
BSA	Bovine Serum Albumin
CO_2	Carbon IV oxide
CDC	Centre of Disease Control
CFU	Colony Forming Units
CHLO(+)	Chloramphenicol (Positive control)
CIPRO (+)	Ciprofloxacin (Positive control)
CMR-KEMRI	Centre of Microbiology Research-Kenya Medical Research Institute
DHFR	Dihydrofolate reductase
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
GC-MS	Gas Chromatography- Mass Spectrometry
H &E	Hematoxylin and Eosin
HSV	Herpes simplex virus

IARC	International Agency for Research on Cancer
IgA	Immunoglobulin A
ICRAF	International Centre for Research in Agroforestry
IZ	Inhibition zone
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KALRO	Kenya Agricultural and Livestock Research Organization
KH_2PO_4	Potassium phosphate
LD_{50}	Median Lethal Dose
Ltd	Limited
MDR	Multi Drug Resistant
MF	Molecular Formula
MIC	Minimum Inhibitory Concentration
MW	Molecular Weight
NADP+	Nicotinamide Adenine Dinucleotide Phosphate (Oxidized)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (Reduced)
NA	No activity
ND	Not Defined
NE	No inhibitory effect
NIST	National Institute Standard and Technology
OECD	Organization of Economic Cooperation and Development

O-H	Oxygen-Hydrogen
PBPs	Penicillin Binding Proteins
PBS	Phosphate-buffered saline
RNA	Ribonucleic acid
PCV	Packed Cell volume
Rf	Retention factor
rRNA	ribosomal Ribonucleic Acid
Rt	Retention time
SD	Standard deviation
TDFE	*Tithonia diversifolia* flower extrac acetate
TDFH	*Tithonia diversifolia* flower extract of hexane
TDLE	*Tithonia diversifolia* leaf extract of ethyl acetate
TDLH	*Tithonia diversifolia* leaf extract of hexane
TDLM	*Tithonia diversifolia* leaf extract of methanol
THF	Tetrahydrofolate
TLC	Thin Layer Chromatography
TMP	Trimethoprim
USA	United State of America
Vi-Ps	Vi Polysaccharides
Vi-TT	Vi-Tetanus Toxoid

WHO	World Health Organization
WURE	*Warburgia ugandensis* root extract of ethyl acetate
WURH	*Warburgia ugandensis* root extract of hexane
WUSBE	*Warburgia ugandensis* stem bark extract of ethyl acetate
WUSBH	*Warburgia ugandensis* stem bark extract of hexane

ABSTRACT

The extracts from *T. diversifolia*, *W. ugandesis*, *C. megalocarpus*, *C. edulis* and *L. cornuta* plants traditionally used in treatment of typhoid fever were screened for anti-salmonella activity using disc diffusion and microdilution techniques. The results from the present study have shown that out of thirty six extracts investigated, only nine extracts from *T. diversifolia* and *W. ugandensis* showed activity against four clinical *Salmonell*a isolates at 1000 mg/ml. The inhibition zones of 9 plant extracts ranged from 8 to 18.5 ± 0 mm. These results were comparable with those of ciprofloxacin (19.67 to 26 mm) and chloramphenicol (6.67 to 24.33 mm). The Minimum Inhibitory Concentration (MIC) of the 9 plant extracts were in the range of 0.031 to 15.63 mg/ml which compared well with ciprofloxacin (0.015 to 0.02) and chloramphenicol (0.022 to 0.03 mg/ml). The safety profiles of the 9 extracts were evaluated for acute and sub-chronic toxicity in Swiss white mice. In acute toxicity study, each extract up to 2000mg/kg given in single dose orally did not produce any toxic effect or death. In sub-chronic toxicity study, administration of 300mg/kg, 600mg/kg and 1200mg/kg to treatment groups for consecutive 28 days twice daily of the plant extracts did not produce any toxic effect or death. No gross lesions and histopathological changes were detectable between controls and the treated animals. The nine extracts that demonstrated *in vitro* anti-*Salmonella* activity were also tested for *in vivo* anti-salmonella activity in mice, experimentally infected with *S. ser. Typhymurium* ATCC 1408. *In vivo* studies showed that only three extracts; methanol extract of *T. diversifolia* leaf, ethyl acetate and hexane extracts of stem bark and root respectively of *W. ugandensis* were observed to have anti-salmonella activity in mice.The extracts of the two plants were fractionated by column chromatography (CC) technique and 7, 4 and 7 fractions were obtained respectively. The MICs values of the CC fractions were determined by microdilution assay. The MIC values of the fractions ranged from 1.22-312.5µg/ml. These results were comparable with that of ciprofloxacin (1.22-19.53µg/ml). In addition, the fractions were also evaluated for their abilities to inhibit dihydrofolate reductase activity. The fractions from *T. diversifolia* and *W. ugandensis* plants showed high potency as anti-salmonella agents and deserve

further investigations in this regard. Gas Chromatography- Mass Spectrometry analysis was carried out to identify compounds in the fractions and a total of thirty three known compounds were identified. For example, hexadecanoic acid, 9, 12- octadecadienoic acid (Z, Z), 1, 2-benzenedicarboxylic, 1, 4, 8-cycloudecatriene-2, 6, 6-carophylene, ß-sequiphellandrene and 3-ethenyl-3-methyl-2, 1-(1-methylethenyl) cyclohexanol were identified and their fractions demonistrated strong anti-salmonella activity. These findings demonstrate that *T. diversifolia* and *W. ugandensis* extracts exhibit appreciable amount of anti-salmonella activity and thus have great potential as a source for natural health products.

CHAPTER ONE

INTRODUCTION

1.1 General introduction

1.1.1 Typhoid fever

Typhoid fever is also known simply as typhoid, is a bacterial infection due to *Salmonella typhi* (Wain *et al*., 2015; Fink & Cookson, 2007). It is among the most common febrile illness encountered by physicians in developing countries. After an incubation period of 7 to 14 days, the onset of bacteremia is marked by fever and malaise. Patients typically present with fever, influenza-like symptom with chills, headache, malaise, anorexia, nausea, poorly localized abdominal discomfort, dry cough and myalgia (Chowdhury *et al*., 2014). Symptoms may vary from mild to severe and usually begin six to thirty days after exposure. The risks of the disease has increased in population exposed to unsafe water and food and also pose a risk to, travellers visiting endemic countries (Crump & Mintz, 2010).

There are approximately 22 million new typhoid cases occurring each year. The worst sufferers are between 5 and 19 years old (Upadhyay *et al*., 2015) in poor, resource- limited areas, who make up the majority of the new cases and mortality figures (215,000 deaths annually). Most of these deaths are due to *S. typhi* infection with south-east Asian countries bear the brunt of the disease. Other areas of prevalence include Africa and South America. Outbreaks have been reported from Zambia, Zimbabwe, Fiji and the Philippines (Upadhyay *et al*., 2015). There is evidence that typhoid fever is often under reported, so the actual figures might be even more than those mentioned above (Darton *et al*., 2014). Historically, in the pre-antibiotic era, the case fatality rate of typhoid fever was 10–20%. Currently, with prompt treatment, the case of fatality rate is less than 1 % (Heymann, 2008). However, about 3-5% of individuals who are infected could develop a chronic infection in the gall bladder (Rajesh *et al*., 2015). Since *S. typhi* is human-restricted, these chronic carriers become the crucial reservoir, which can persist for decades risking further spread of the disease. This also complicates the identification and treatment of the disease (Gonzalez *et al*., 2010).

Figure 1.1 shows global distribution pattern of typhoid fever incidences.

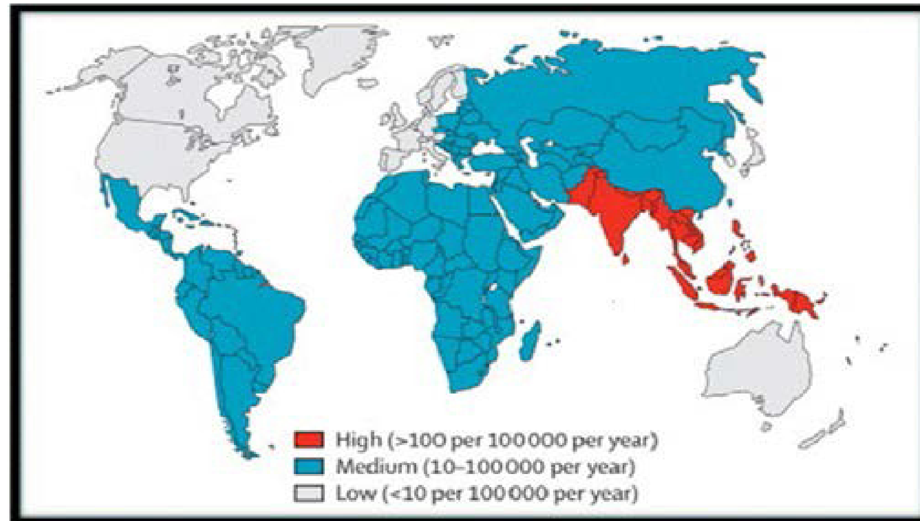


Figure 1.1: Global distribution of typhoid fever

Source: Crump and Mintz (2010)

The social and economic impact of typhoid fever is also high because patients with acute disease and complications may need to be hospitalized. This results in loss of work days and, consequently, income (Background document, WHO, 2015).

There are several factors, which are associated with relapse of culture proven typhoid fever as seen over 15 years in 1,650 children in Multi Drug Resistant (MDR) isolates in South Asia. Despite the drop in morbidity and mortality associated with typhoid fever due to the advent of antibiotics, relapses continue to occur in up to 10% of the patients, even though they are immunocompetent. Patients with drug-resistant typhoid fever who received ineffective therapy have a relapse rate, which is almost twice that of those infected with sensitive isolates. Diarrhea is associated with lower relapse rates in children infected with pan-sensitive typhoid fever. Those infected with MDR isolates have a higher relapse rate when presenting with constipation or starting specific therapy within 14 days of fever onset. The use of quinolones or cephalosporins as part of the treatment course protects against subsequent relapse. In those areas where MDR typhoid

fever caused by *S. typhi* is prevalent, empirical treatment of patients with cephalosporin or quinolone should be considered, until infection is caused by a drug-sensitive strain (Ahmad *et al*., 2011).

Salmonella typhi organism is a gram-negative bacterial pathogen that infects humans, causing significant morbidity and mortality worldwide (Fink & Cookson, 2007). The bacterium is serologically positive for lipopolysaccharide antigens O9 and O12, protein flagellar antigen Hd, and polysaccharide capsular antigen Vi. The Vi capsular antigen is largely associated with *S. enterica serotype typhi*. Polysaccharide capsule Vi has a protective effect against the bactericidal action of serum of infected person (Background document, WHO, 2003).

Salmonella typhi attach themselves to the epithelial cells of the small intestines, penetrate the sub- mucosa, and pass from there into the blood stream via the lymphatics. A transient bacteremia follows and the *S. typhi* enter the recticulo-endothelial system (liver, spleen, bone marrow) and the gall bladder and kidneys. *Salmonella typhi* re-enter the intestine from the gall-bladder where it involves the Peyers patches, and least inflammation and ulceration. The incubation period is about 5 to 21 days (Kaur & Jain, 2012).

1.1.2 Prevention and Control strategies of typhoid fever

Contaminated water and food are important vehicles for transmission of typhoid fever. Historical surveillance data suggest that typhoid fever was endemic in Western Europe and North America and that rate decreased in parallel with the introduction of treatment of municipal water, pasteurization of dairy products, and proper disposal of contaminated foods. At present, typhoid fever prevention focuses on improving sanitation, ensuring the safety of food and water supplies, identification and treatment of chronic carriers of *S. typhi*, and use of typhoid vaccines to reduce the susceptibility of hosts to infection (WHO, 2011).

Extending the benefits of improved sanitation and the availability of safe water and food that was achieved in industrialized countries a century ago to low and middle income countries has proved to be a challenge. United Nations Sustainable Development Goal number 7 sets a target to halve, update, the proportion of

population without sustainable access to safe drinking water and basic sanitation. It has been suggested that interventions to improve the quality of drinking water is relatively more important for the prevention of typhoid infection relative to sanitation measures than was previously thought (Clasen *et al*., 2007). Although centrally treated reticulated water for all is an important goal, a growing body of research suggests that improving water quality at the household level, as well as at the source, can significantly reduce diarrhea (Clasen *et al*., 2007). Although not formally evaluated with typhoid fever as an outcome, it is generally accepted that interventions that reduce the rate of diarrheal diseases transmitted through contaminated water, food, and poor hygiene would have similar effects on rates of enteric fever.

The identification and treatment of *S. typhi* carriers, particularly those involved with food production, has proven to be an important strategy for the control of typhoid fever in low incidence settings. Although carriers can be identified by serial culture of stool specimens, this approach is labor intensive. Anti-Vi antibody assays have proven to be a useful alternative to stool culture for identifying carriers in outbreak settings, however, rectal swabs can be obtained instead of stool samples but they are less successful in isolating the *S. typhi* (Background document, WHO, 2015).

Three types of typhoid vaccines are currently available: Phenol-inactivated vaccine; Live, attenuated *S. typhi* strain, Ty21a; Purified Vi capsular polysaccharide vaccine. As of now, only two types of typhoid vaccines are reportedly available in the Indian market for use clinically, namely the Vi polysaccharide (Vi-PS) vaccine and the Ty21a oral vaccine (Milind *et al*., 2015). Each of these vaccines offers 55% to 85% protection for 3 to 5 years. The main differences relate to their side effects. Local pain at the injection site and mild to moderate systemic reactions are commonly encountered with the phenol-inactivated vaccine. The live-attenuated oral vaccine may cause mild gastrointestinal distress, but because of its low toxicity and ease of administration it should be used for travellers to areas of high risk (Zuckerman *et al*., 2007). There is little data available regarding the protective efficacy of the oral vaccine for travellers. The purified capsular Vi vaccine has significantly fewer adverse effects than the killed whole cell parenteral vaccines. An

effective typhoid vaccine could have a substantial effect during outbreaks in locations where water and sewage-disposal systems are inadequate. Although typhoid fever is common in India, and there are concerns about the prevalence of multidrug resistant isolates, the typhoid vaccine is being grossly underutilized (Upadhyay *et al*., 2015). The use of vaccines appear to be cost effective, considering the financial implications of diagnosing typhoid by blood culture, the expenditures on hospitalization and medicines, and the loss of daily productive working hours, as a result of illness. Therefore, the expert group recommends the use of available vaccines routinely in unvaccinated adults, especially those who are at high risk (Murugunathan *et al*., 2015). The Vi-Tetanus Toxoid (Vi-TT) conjugate vaccine is a fourth generation typhoid vaccine that has been indigenously developed by an Indian biotechnological company. After being tested and analyzed for efficacy and safety in more than a thousand individuals belonging to different age groups, this vaccine was launched in Hyderabad in 2013 (Szu, 2013). As evident from the four fold increase in the serum IgA responses of patients, the vaccine evoked a seroconversion of 98% in infants between 6 and 24 months of age, 99% in children aged 2 to 15 years, and 92% in individuals belonging to the 15-45 year age group. It has been shown to be superior to the Vi-PS typhoid vaccines and also has a good safety profile, being tolerated by people of all the tested age groups (Murugunathan *et al*., 2015).

Treatment of typhoid fever has been complicated by the development and rapid dissemination of typhoidal *Salmonella* isolates resistant to ampicillin, trimethoprim-sulfamethoxazole, and chloramphenicol (i.e., multidrug-resistant or MDR isolates). In recent years, development of resistance to fluoroquinolones has resulted in more challenges (Rupnik *et al*., 2009). The mainstay of typhoid fever management is the use of antibiotics for empiric or directed therapy. Improper use of antibiotics, especially broad-spectrum antibiotics can lead to emergence of resistance. The commonest factors that lead to antibiotic resistance are the misuse and overuse of these drugs (Khandeparkar, 2010). A re-emergence of chloramphenicol susceptibility in *S. enteric serovar typhi* isolates has been witnessed in some regions of India, where the susceptibility has been found to be as high as 95%. Investigators have suggested using chloramphenicol, along with the

third-generation cephalosporins in typhoid fever due to ciprofloxacin-resistant *S. enteric serovar typhi* infection (Harish *et al*., 2015). Resistance to fluoroquinolones has led to an increased use of azithromycin and third-generation cephalosporins. There are worldwide reports of high level resistance to expanded spectrum cephalosporins (e.g. ceftriaxone). Spread of such resistance would further limit the available therapeutic options, with only reserve antimicrobials like carbapenem and tigecycline left as possible treatment options (Harish *et al*., 2015). It is suggested that quinolones and third-generation cephalosporins be used as first-line antimicrobials in typhoid fever. It has been suggested that the use of fourth- generation cephalosporins should be restricted to complicated or resistant cases (Vala *et al*., 2014).

1.1.3 Challenges associated with typhoid fever management

Evidence suggests that the fluoroquinolones and tetracycline are the optimal choice for combating typhoid fever in adults and that they may also be used in children (Malik & McBride, 2015). However, *Salmonella* isolates resistant to these antibiotics have been reported in Korea and other countries (Stevenson *et al*., 2007). One major concern to public health has been the global dissemination of *S. typhimurium* Definitive Type 104, which is resistant to cotrimoxazole, nalidixic acid and ampicilin (Perron *et al*., 2008; Kariuki *et al*., 2010). In addition, the recent emergence of resistance to fluoroquinolones, due to indiscriminate and widespread use in primary health care settings is threatening. In addition to resistance problem, the safety of antibiotics remains an enormous global issue. It was estimated that 2.22 million hospitalized patients had serious Adverse Drug Reactions (ADR) and 106,000 died in a single year in the USA (Fox, 2011).

Mechanisms of action of current chemotherapeutics of typhoid fever have been reported. For instance, it has been reported that cefixime inhibits bacterial cell wall synthesis by binding to one or more of the penicillin binding proteins (PBPs); which in turn inhibit the final transpeptidation step of peptidoglycan synthesis in bacterial cell walls, thus inhibiting cell wall biosynthesis (Zapun *et al*., 2008). Bacterial membrane eventually lyse due to ongoing activity of cell wall autolytic enzymes (autolysins and murein hydrolases) while cell wall assembly is arrested (Mayer,

2010). Likewise, chloramphenicol inhibits peptidyl transferase activity of the bacterial ribosome. It binds to A2451 and A2452 amino residues in the 23S rRNA of the 50S ribosomal subunit and prevents peptide bond formation (Schifano *et al*., 2013; Mayer, 2010). Ciprofloxacin, considered a benchmark when comparing new fluoroquinolones, shares with these agents a common mechanism of action thus by inhibiting DNA gyrase, and a type II topoisomerase, topoisomerase IV, (East & Silver., 2013) necessary to separate bacterial DNA, thereby inhibiting cell division. While ciprofloxacin demonstrated a fairly good activity against gram-positive bacteria, it is against gram-negative organisms that it proved to be more potent than other fluoroquinolones (Simoens *et al*., 2010). Meanwhile Trimethoprim, Methotrexate and Pyrimethamine bind to dihydrofolate reductase and inhibit formation of tetrahydrofolic acid. In biological systems, DHFR enzyme catalyzes the formation of tetrahydrofolate by reduction of dihydrofolate using NADPH as a cofactor (Abali *et al*., 2008). The antibiotics have broad range of activity against gram-positive and gram-negative bacteria and are used in combination with sulfonamides; this combination blocks two distinct steps in folic acid metabolism and prevents the emergence of resistant isolates (Gallo *et al*., 2013).

In general, despite advances in technology and public health strategies, typhoid fever remains a major cause of morbidity in the developing world. In some areas typhoid fever disproportionately affects young children and may reflect high rates of transmission through food and water. Recent emergence of drug resistance especially to common, first line antibiotics and quinolones has made it very difficult and expensive for health services to manage the disease. Rapid and appropriate diagnostics are key to the management of typhoid in terms of public health. Although effective vaccines are available; there are no plans for large scale vaccination programmes in infants and children.

1.1.4 The role of Dehydrofolate reductase enzyme (DHFR) in *S. typhi*

In biological systems, DHFR enzyme catalyzes the formation of tetrahydrofolate by reduction of dihydrofolate using NADPH as a cofactor. Tetrahydrofolate and its one carbon adducts are required for *de novo* synthesis of purines and thymidylate, as well as some amino acids. DHFR inhibition causes disruption of purine and thymidylate

biosynthesis and DNA replication, leading to cell death (Abali *et al*., 2008). Therefore, DHFR has been an attractive target for chemotherapy of many diseases including cancer (Gonen & Assaraf, 2012), malaria (Anderson, 2005), leishmania and trypanosomiasis (Gilbert, 2002) and bacterial infections (Hawser *et al*., 2006).

1.1.5 Ethnopharmacological uses of selected medicinal plants

Interest in medicinal plants as a re-emerging health aid has been fuelled by the rising costs of prescription drugs in the maintenance of personal health and well-being and bioprospecting of new plant derived drugs. Based on current research and financial investments, medicinal plants will seemingly; continue to play an important role as a health aid. Plants used in this study have traditionally been associated with disease curative and preventive practices in many countries for a long time. Garcia and Delgado (2006) reported that *Tithornia diversifolia* has promising medicinal value. Skin products formulated from *T. diversifolia* extracts have been shown to have antimicrobial properties (Kareru *et al*., 2010). In Ethiopia *Warburgia ugandensis* extracts are used to treat malaria, tuberculosis, bronchitis, pneumonia, hepatitis and gonorrhea (Wube *et al*., 2010; Were *et al*., 2010; Opiyo *et al*., 2011). The decoction from *Croton megalocarpus* bark is used as a remedy for whooping cough and pneumonia (Kariuki *et al*., 2014). Grounded roots are used for syphilis and anthrax treatment (Kabir *et al*., 2005). Different communities in Africa use parts of *Carissa edulis* to alleviate pain, treat venereal diseases and glandular inflammation (Githiori *et al*., 2004). In Kenya and Tanzania decoction from *Launae cornuta* roots is used as a remedy for cough, typhus fever and measles (Schippers, 2004).

This study investigated the anti-salmonella activities of *T. diversifolia*, *W. ugandensis*, *C. megalocarpus*, *C. edulis* and *L. cornuta*. Clinical isolates of *S. ser. Typhi* (ATCC 13347), *S. ser. Typhi* (ATCC 43579), *S. enterica* (ATCC 2162) and *S. ser. Typhimurium* (ATCC 1408) were used in the study.

1.2 Statement of the problem

Typhoid fever continues to be a major health problem despite the use of antibiotics and the development of newer anti-salmonella typhi drugs. The current emergence and re-emergency of MDR isolates against fluoroquinolones, the spread of MDR and fluoroquinolone resistance in *S. typhi* presents significant clinical challenges. Typhoid in Kenya is associated with a dominant Multidrug-Resistant *Salmonella enterica serovar typhi* Haplotype that is also widespread in Southeast Asia. In addition, Side effects (anolexia, headache,) and treatment failure of conventional drugs especially fluoroquinolones has been reported in India. It is estimated that 2.22 million hospitalized patients had serious Adverse Drug Reactions (ADR) and 106,000 died in a single year in the USA. Available drugs are very expensive and limited to the infected patients with typhoid fever. Hence, there was need to bio-prospect for alternative anti-salmonella agents from the selected Kenyan medicinal plants for treatment of typhoid fever.

1.3 Justification of the study

World-wide, medicinal plants continue to be relied upon as solutions to health problems, majority of them use plants or their active principles. Natural biological active compounds in plants have a significant role in plants defense mechanism and also important for their unambiguous physiological action in human body. Because of the therapeutic property, secondary metabolites (flavonoids, alkaloids, tannins and terpenoidal saponins) are becoming a part of the integrative health care system as supportive and alternative medicines. For the cure of infectious diseases the unsystematic use of commercial antimicrobial medicines has led to development of numerous drug resistances in human pathogenic microorganisms. Besides, a number of side effects like hypersensitivity, allergic reactions and immune suppression are rarely associated with the antibiotics. Further, food preservation requires evaluation of natural resources such as herbal fractions and isolates with antimicrobial properties as the long historic use of herbs has proved their safety and efficacy in various traditional medicine systems. Recent trends for the use of natural remedies as antimicrobial have increased their use in food, cosmetic and pharmaceutical products which have been screened *in vitro* and indicated antimicrobial and other diverse

properties. Novel compounds have a vast therapeutic ability, lessen various adverse effects, and are often coupled with synthetic antimicrobials.

Moreover, many plants are used in Africa continent including Kenya for treatment of different diseases such as fever, dysentry, Cholera diarrhea etc and others which are typical diseases of a tropical country. Medicinal herbs with unique chemical compounds that can either inhibit the growth of pathogens or kill them and have no or least toxicity to host cells are considered as potential candidates for developing new antimicrobial drugs. Traditional practitioners use herbal preparations to treat microbial infections such as typhoid and paratyphoid infections because they claim that they are relatively safer than synthetic alternatives, offers profound therapeutic benefits and they are more affordable for treatments. The aim of this study was to verify whether the claimed anti-salmonella properties of *T. diversifolia*, *W. ugandensis*, *C. megalocarpus*, *C. edulis* and *L. cornuta* plants used by traditional practitioners can be scientifically confirmed.

1.4 Null hypothesis

Extracts from *T. diversifolia*, *W. ugandensis*, *C. megalocarpus*, *C. edulis* and *L. cornuta* have no anti-salmonella activity.

1.5 Objectives

1.5.1 Broad objective

To analyse *T. diversifolia*, *W. ugandensis*, *C. megalocarpus*, *C. edulis* and *L. cornuta* plant extracts for their bioactivity against the 4 clinical *Salmonella* isolates.

1.5.2 Specific objectives

1. To determine phytochemical constituents in *T. diversifolia*, *W. ugandensis*, *C. megalocarpus*, *C. edulis* and *L. cornuta* extracts.
2. To evaluate *in vitro* activity of extracts of *T. diversifolia*, *W. ugandensis*, *C. megalocarpus*, *C. edulis* and *L. cornuta* plants against *S. ser. Typhi* (ATCC 13347), *S. ser. Typhi* (ATCC 43579), *S. enterica* (ATCC 2162) and *S. ser. Typhimurium* (ATCC 1408).
3. To assess safety levels of identified plant extracts in mice subjects.
4. To determine efficacy of the extract(s) in mice infected with *S. ser. Typhimurium.*
5. To evaluate *in vitro* anti-salmonella activity of the column chromatography fractions of selected plants and their effect on *S. ser.Typhimurium* DHFR activity.
6. To identify the compound(s) in the anti-salmonella active plant fractions.

CHAPTER TWO

LITERATURE REVIEW

2.1 Clinical *Salmonella typhi* and its impacts

Salmonella typhi is an important pathogen that causes an estimated 1.4 million illnesses, 16,000 hospitalizations, and between 400 and 600 deaths annually in the United States alone (Cummings *et al*., 2010). Although primarily a cause of self-limiting acute enteritis (diarrhea, abdominal pain, and fever, with a typical duration of 4 to 7 days), *Salmonella* can produce invasive infections that lead to sepsis and death (Feasey *et al*., 2012). Young children, the elderly, and those with compromised immune systems are especially susceptible to severe disease. The prevalence of multidrug resistance among *Salmonella* isolates has increased over the past two decades, making treatment failures more common among those with serious disease. In addition, infections with resistant isolates of *Salmonella* tend to be more severe and lead to higher rates of hospitalization than those caused by susceptible isolates (Kariuki *et al*., 2006; Varma *et al*., 2005a, 2005b).

For more than 40 years since its discovery, chloramphenicol was the drug of choice for the treatment of typhoid (Butler, 2011). However, the emergence in the late 1980s of MDR *serovar typhi* (isolates resistant to ampicillin, chloramphenicol, and cotrimoxazole) in outbreaks reported in the Indian subcontinent (Kanungo *et al.,* 2008), Arabian Gulf, the Philippines, and South Africa (Kawser *et al*., 2013) led to the use of the fluoroquinolones as alternative drugs (Kawser *et al*., 2013). Among the first reports of clinical treatment failure due to *serovar typhi* resistant to nalidixic acid and showing then an increased ciprofloxacin MIC (0.125 µg/ml) was in 1991 in a patient who had returned to the United Kingdom from India (Tatavarthy *et al*., 2014). Thereafter, several cases of MDR *serovar typhi* also resistant to nalidixic acid and the fluoroquinolones have been reported in Bangladesh, India (Tatavarthy *et al*., 2014), raising concerns about further spread to other regions where typhoid fever is endemic.

Consequent on the current emergence and re-emergence of resistant isolates of most microorganisms, coupled with side effects of most conventional drugs, interest in the

use of plant and plant products in the management of ailments is increasing. Indeed, nature has remained a veritable source of medicinal agents since ancient times (Babayi *et al*., 2004). Although the *in vitro* and *in vivo* properties of some medicinal plants to microbial pathogens have been widely reported (Gomaa & Hashish, 2002; Iwalokun *et al*., 2004), most are actually yet to receive any scientific backing. Therefore, the present study was to verify whether the claimed anti-salmonella properties of *T. diversifolia*, *W. ugandensis*, *C. megalocarpus*, *C. edulis* and *L. cornuta* plants by traditional practitioners can be scientifically confirmed and provide support for their use in the treatment and management of typhoid fever.

2.2 Plants with antibacterial activity

Currently, there has been considerable interest in the use of plant materials as an alternative method of controlling pathogenic microorganisms (Inácio *et al*., 2013), and many compounds from plants have been shown to be effective against resistant pathogenic bacteria (Samy *et al*., 2013). Worldwide, medicinal plants are the best sources to obtain a variety of new herbal drugs. About 80% of individuals from developing countries use traditional medicine, which has substances derived from medicinal plants (Sahoo *et al*., 2010; Verma & Singh, 2008). Some of plants have been reported to have antibacterial activity. For instance, *Euphorbia prostrata* (*E. prostrata*) is an annual herb, which belongs to family Euphorbiaceae and is abundantly found in India and Africa. It has been traditionally used in several digestive system disorders (Baptista *et al*., 2013; Gupta, 2011). In Burkina Faso, the leaves are used as a remedy against the bites of venomous insects (wasps, scorpions). In Togo, this plant is used to treat infertility and menstrual pain (Schmelzer & Gurib-Fakim, 2008) and in the western rural parts of Cameroon; the whole plant of *E. prostrata* is frequently used for the treatment of dysentery and typhoid fever (Husain *et al*., 2008). The *in vitro* antimicrobial activity of *E. prostrata* extracts against *S. typhi*, *S. paratyphi* A, *S. paratyphi* B and *S. typhimurium* has been reported by Kengni *et al*., (2013). It has been demonstrated that the aqueous extract of *E. prostrata* is considered as practically non- toxic, since the LD_{50} values of the extract were found to be 23.2 g/kg and 26.4 g/kg for

female and male mice, respectively (Kengni *et al*., 2013). These LD50 values were greater than 5 g/kg, as stated by the Hodge and Sterner criteria (Ouédraogo *et al*., 2011).

Anti-salmonella activity of *Terminalia belerica*, an ingredient of Ayurvedic preparation 'triphala' used for treatment of digestive and liver disorders, has been reported (Midan & Jain, 2008). Fruits of *T. belerica* were extracted with petroleum ether, chloroform, acetone, alcohol and water and efficacy of extracts against *Salmonella typhi* and *Salmonella typhimurium* was evaluated. Alcoholic and water extracts of *T. belerica* showed significant anti-salmonella activity and MIC of 12.5 mg/ml against *S. typhimurium* (Midani & Jain, 2008). Aqueous extracts of *Picrohiza kurroa* and *Vitits vinefera* also showed low anti-salmonella activity where as aqueous extracts of *Asparagus racemosus* and *Zingiber officinale* showed no anti-salmonella activity (Ewam, 2014). Extracts of *T. belerica*, *Picrohiza kurroa* and *Vitits vinefera* with other solvents such as chloroform and petroleum ether showed insignificant activity. It has been reported that aqueous extract of *T. belerica* has bactericidal activity at high concentrations where as low concentrations has bacteriostatic property. *In vitro* cellular toxicity studies showed no cyto-toxicity associated with *T. belerica* extracts. Pretreatment of mice with aqueous extract of *T. belerica* conferred protection against experimental salmonellosis and 100% survival of animals has been reported when challenged with lethal doses of *S. typhimurium* (Midani & Jain, 2008).

2.3 Secondary metabolites from plants with antibacterial activity

Plant-derived compounds of therapeutic value are mostly secondary plant metabolites traditionally used for medicinal purposes. They have a wide activity range, according to the species, the topography and climate of the country of origin, and may contain different categories of active principles (Assob *et al*., 2011; Arruda *et al*., 2011; Ahmad *et al*., 2006). Variations in the chemical composition modify their antimicrobial activity. Some main categories of phytochemicals extracted from medicinal plants which have been reported to have pharmacological activities include:

2.3.1 Flavonoids

Flavonoids, previously called bioflavonoids and included in aromatic compounds, are phenolic structures ubiquitous in photosynthesizing cells and are commonly found in fruit, vegetables, nuts, seeds, stems, flowers, tea, wine, propolis and honey (Temitope, 2015). For centuries, preparations containing these compounds as the principal physiologically active constituents have been used to treat human diseases. The basic structural feature of flavonoid compounds is the 2-phenyl-benzopyrane or flavane nucleus, consisting of two benzene rings linked through a heterocyclic pyrane ring. Flavanoids are a family of plant-derived compounds with potentially exploitable antibacterial activities, including direct antibacterial activity or synergistic flavonoid–antibiotic combinations activity (Cushnie & Lamb, 2011).

In total, there are 14 classes of flavonoids, differentiated on the basis of the chemical nature and position of substituents on the different rings. Catechin (Figure 2.1), the most reduced form of the C_3 unit in flavonoid compounds, deserve special mention. These flavonoids have been extensively researched due to their occurrence in oolong green teas. It was noticed some time ago that teas exerted antimicrobial activity and that they contain a mixture of catechin compounds. These compounds inhibited in vitro *Vibrio cholerae* O1, *Streptococcus mutans Shigella*, and other bacteria and microorganisms (Ullah & Khan, 2016). The antibacterial properties of flavonoids are thought to come from the ability to form complexes with both extracellular and soluble proteins, as well as with bacterial membranes (Fowler *et al*., 2011).

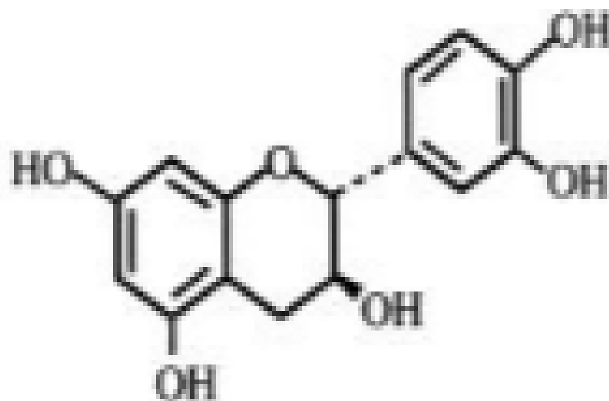


Figure 2.1: Chemical structure of catechin

Kuete, (2011), demonstrated that among the flavonoids hydroxylating the prenyl groups of stipulin, the compounds obtained angusticornin B and bartericin A, had a superior antimicrobial activity. Thus, the prenyl group plays an important role in the activity of chalcones. Recently two flavonoids (6-hydroxy-7-methoxyluteolin and the xanthone 8-carboxymethyl- 1, 5 ,6-trihydroxy-3-methoxyxanthone) extracted from the leaves of *Leiothrix spiralis*, a South American plant belonging to the Eriocaulaceae family, showed a promising activity on *Escherichia coli* and *Pseudomonas aeruginosa* (Araujo *et al*., 2011). Some flavonoids also revealed activity against *M. tuberculosis* (Garcia *et al*., 2012).

A synergy has been demonstrated between active flavonoids as well as between flavonoids and existing chemotherapeutics, even if the reports of activity in the field of antibacterial flavonoid research are widely conflicting, probably owing to inter- and intra-assay variation in susceptibility testing (Cushnie & Lamb, 2011). Future optimization of these compounds through structural alteration may allow the development of a pharmacologically acceptable antimicrobial agent or group of agents. Existing structure–activity data suggest that it might be possible, for example, to prepare a potent antibacterial flavanone by synthesizing a compound with halogenation of the B ring as well as lavandulyl or geranyl substitution of the A ring (Atta *et al*., 2013). Also, it is worth noting that by elucidating flavonoid biosynthetic pathways it would be possible to produce structural analogs of active flavonoids through genetic manipulation. Numerous research groups have sought to elucidate the antibacterial mechanisms of action

of selected flavonoids; the activity of quercetin has been at least partially attributed to the inhibition of DNA gyrase, whereas sophoraflavone G and epigallocatechin Galati inhibit cytoplasmic membrane function, and licochalcones A and C inhibit energy metabolism (Chikezie *et al*., 2015).

2.3.2 Alkaloids

Alkaloids are heterocyclic nitrogen compounds characterized by different antimicrobial activities. The analysis of the leaf extracts of *Gymnema montanum* and of ethanol extract of *Tabernaemontana catharinensis* root bark revealed an antimicrobial activity (Ramkumar *et al*., 2007; Medeiros *et al*., 2011). In the first case it was due to an activity depending upon the chemical composition of the extracts and membrane permeability of the microbes, and in the second case was linked to indole alkaloids responsible for the observed antibacterial and antidermatophytic activity. Diterpene alkaloids, commonly isolated from the plants of the Ranuncolaceae group, had antimicrobial properties (Savoia, 2012). Berberine (Figure 2.2), an isoquinoline alkaloid, present in roots and stem-bark of Berberis species, is a hydrophobic cation widely used in traditional medicine owing to its activity against bacteria, fungi, protozoa and viruses (Sabo, 2015). It accumulates in cells driven by the membrane potential and is an excellent DNA intercalator (Ball *et al.,* 2006), active on several microorganisms with a target on RNA polymerase, gyrase and topoisomerase IV and on nucleic acid (Yi *et al.,* 2007).

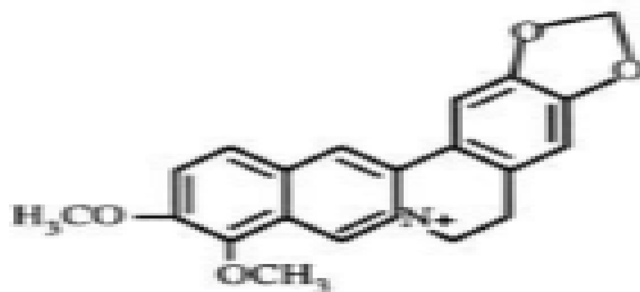


Figure 2.2: Chemical structure of barberine

2.3.3 Terpenes

Terpenes compounds are also referred to as isoprenoids and their derivatives containing additional elements, usually oxygen, are called terpenoids. A terpenoid constituent, capsaicin (Figure 2.3), has a wide range of biological activities in humans, affecting the nervous, cardiovascular, and digestive systems as well as finding use as an analgesic (Bazzo *et al*., 2012). The evidence for its antimicrobial activity is mixed. Cichewicz and Thorpe (1996) found that capsaicin might enhance the growth of *Candida albicans* but that it clearly inhibited various bacteria to differing extents (Sahu *et al*., 2011). The antibacterial activity of some monoterpenes (C10), diterpenoids, sesquiterpenes (C15), triterpenoids and their derivatives were recently reviewed (Kurek *et al*., 2011). The results obtained illustrate the strong structure– function influence of the antibacterial potential of terpenes. Diterpenoids, such as sesquiterpenes, isolated from different plants exhibited bactericidal activity against Gram-positive bacteria and inhibited the growth of *M. tuberculosis* (Garcia *et al*., 2012; Kurek *et al*., 2011). The mechanism of action of terpenoids is not fully understood, but is speculated to involve membrane disruption by the lipophilic compounds (Termentzi *et al*., 2011).

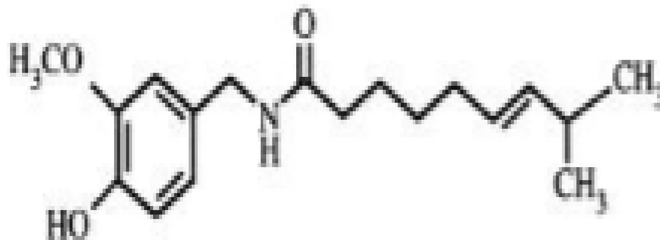


Figure 2.3: Chemical structure of capsaicin

2.3.4 Phenolics and Polyphenols

Phenolic compounds are widely distributed in plants, where they protect the plants from microbial infections. They have potential anti-oxidative properties but are also potent anti- infectives (Saleem *et al*., 2010). They are a large group of aromatic compounds, consisting of flavones, flavanoids and flavanols containing one carbonyl group, quinones with two carbonyl groups, tannins, polymeric

phenolic substances, and coumarins, phenolic compounds with fused benzene and pyrone groups (Kurek *et al*., 2011). Phenolic compounds possessing a C_3 side chain at a lower level of oxidation and containing no oxygen are classified as essential oils and often cited as antimicrobial as well. Eugenol (Figure 2.4) is a well-characterized representative found in clove oil. Eugenol is considered bacteriostatic against both fungi and bacteria (Oussalah *et al*., 2007).

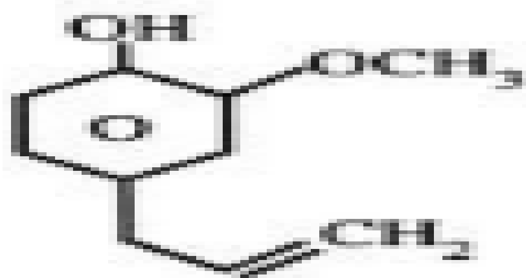


Figure 2.4: Chemical structure of eugenol

2.3.4.1 Flavones

Flavones (Figure 2.5) and their derivatives represent an antibacterial therapeutic possibility to disrupt bacterial envelopes (Cazarolli *et al*., 2008). The catechins are included among the flavan-3-ols or flavanols, present in different plants, particularly in tea-plant *Camelia sinensis*, where they form complexes with the bacterial cell wall and are active on intestinal microorganisms (Friedman *et al*., 2006). Biological assays indicated the inactivation of specific bacterial enzymes by several of these compounds. Moreover significant synergy was also observed between theaflavin and epicatechin against important nosocomial Gram-negative pathogens (Betts *et al*., 2011).

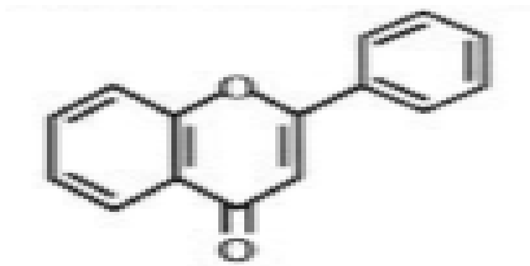


Figure 2.5: Chemical structure of flavone

2.3.4.2 Quinones

Quinones (aromatic rings with two ketone substitutions), ubiquitous in nature, are another significant group of secondary metabolites with potential antimicrobial properties. They provide a source of stable free radicals and irreversibly complex with nucleophilic amino acids in microbial proteins determining loss of their function (Saleem *et al*., 2010). Anthraquinones(Figure 2.6) in particular had a large spectrum of antibacterial (also antimycobacterial) activity, based on inactivation and loss of function of bacterial proteins, such as adhesins, cell wall polypeptides and membrane-bound enzymes (Kurek *et al*., 2011), consequently leading to the death of the pathogens.

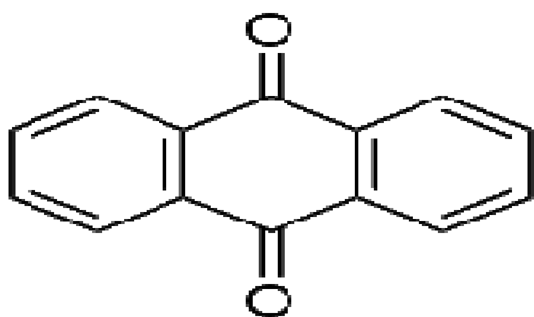


Figure 2.6: Chemical structure of anthraquinones

Hypericin (Figure 2.7) is a naphthodianthrone, a red-colored anthraquinone-derivative, which, together with hyperforin, is one of the principal active constituents of *Hypericum* (Saint John's wort)(Klemow *et al*., 2011). Hypericin is believed to act as an antibiotic, antiviral and non-specific kinase inhibitor. Hypericin may inhibit the action of the enzyme dopamine β-hydroxylase, leading to increased dopamine levels, although thus possibly decreasing norepinephrine and epinephrine.

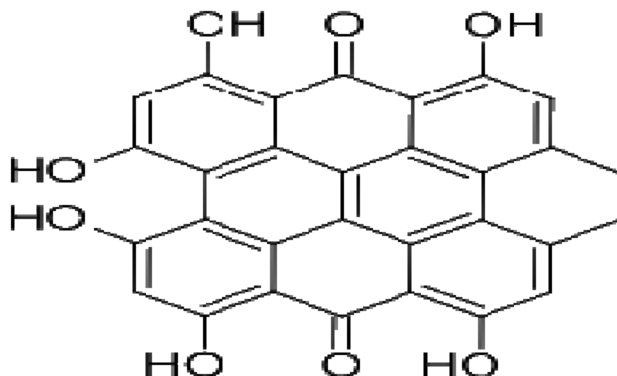


Figure 2.7: Chemical structure of hypericin

2.3.4.3 Tannins

Tannins are a group of polymeric phenolic substances found in almost every plant part characterized by antibacterial activity owing to inactivation of bacterial adhesins, enzymes, and cell envelope and transport proteins. Recently, gallotannin-rich plant extracts demonstrated inhibitory activities on different bacteria attributable to their strong affinity for iron and to the inactivation of membrane-bound proteins (Engels *et al*., 2011).

Hydrolysable and condensed tannins, derived from flavanols, and called proanthocyanidins (Figure 2.8), exert antimicrobial activity by antiperoxidation properties inhibiting in particular the growth of uropathogenic *E. coli* (Kurek *et al*., 2011; Cimolai & Cimolai, 2007). Anthocyanidin synthesis occurs in plants on the cytoplasmic leaflet of the endoplasmic reticulum and then accumulates in the large central vacuole (Cisowska *et al*., 2011). In many plants, anthocyanidins might occur in oligomeric form and in this case they are called proanthocyanidins. Depending on the type of bond between the oligomer-forming anthocyanidin

molecules, two general types (A and B) of proanthocyanidins are distinguished. In less common A-type proanthocyanidins, two bonds are formed between 2β-7 and 4β-8 carbon of oligomer-forming molecules; in B-type, only one 4β-8 bond is formed. The beneficial effects of anthocyanins on human health have been known at least from the 16th century, when blackberry juice was used in the treatment of mouth and eye infections. However, only few studies have focused on the antimicrobial activity of these compounds. Recently, Cisowska *et al*., (2011), described the anthocyanin profile of action of different fruits, mainly berries, but also red grapes and, by consequence, red wine, also containing stilbenoid resveratrol, indicating a superior activity against Gram-positive bacteria (Cisowska *et al*., 2011).

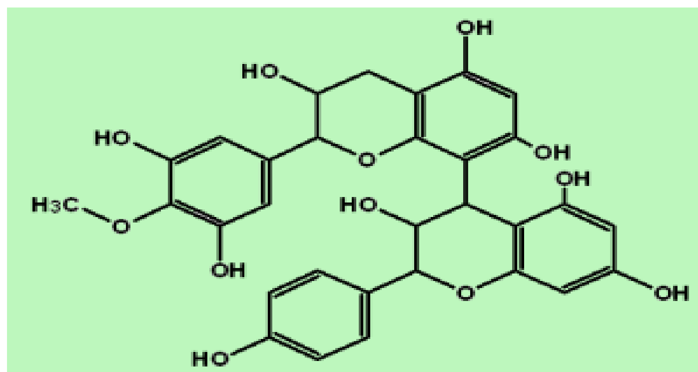


Figure 2.8: Chemical structure of proathocyanidin

Source: www.phytochemicals.info

2.3.5 Coumarins

One known coumarin, scopoletin (Figure 2.9), and two chalcones were isolated as antitubercular constituents of the whole plant *Fatoua pilosa* (Garcia *et al*., 2012). Also, spices and aromatic plants have an antimicrobial effectiveness that depends on the kind of plant, its composition and concentration of essential oils, often rich in monoterpenes and sesquiterpenes (Sokovic *et al*., 2010; Militello *et al*., 2011; Mothan *et al*., 2011; Rahman *et al*., 2011). Studies analyzing the antimicrobial activity of essential oil of *Allium sphaerocephalon* inflorescenses (Lazarevic *et al*., 2011) revealed the accordance with the popular use of plants belonging to the

Allium genus in traditional medicine, indicating the importance of aroma precursors (cysteine sulfoxides) for a potent biologic activity.

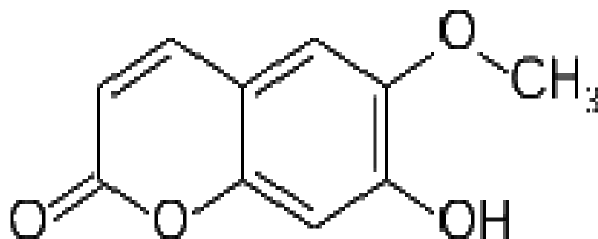


Figure 2.9: Chemical structure of scopoletin

Although, positive anti-salmonella activity has been reported on various plants extracts and natural products mentioned above, there was need to focus and investigate the efficacy of *T. diversifolia*, *W. ugandensis, C. megalocarpus*, *C. edulis* and *L. cornuta* extracts for treatment of typhoid fever.

2.4 Medicinal properties and uses of the five selected plant species

2.4.1 *Tithonia diversifolia*

Tithonia diversifolia (Hemsley) A. Gray commonly known as Mexican sunflower or Mexican arnica is a member of the family Asteraceae. It is an annual weed growing aggressively in abandoned lands, road-sides, river banks and cultivated farmlands. The plant is adaptable to most soils (Olabode *et al*., 2007). Though a native of Central America, it has become naturalized in many tropical countries including the North-East regions of India. In Manipur the plant is widely grown in wild, especially at the foothills and roadsides. There had been reports of *Tithonia* being used for a wide variety of purposes including medicinal and ethnobotanical values (Garcia & Delgado, 2006). Scientific research has established that skincare products formulated from *T. diversifolia* extracts had remarkable antimicrobial properties (Kareru *et al*., 2010). It has been reported that dichloromethane leaf and flower extracts of *T. diversifolia* have antiplasmodial (Muganga *et al*., 2010). In general, the genus *Tithonia* is an important source of diverse natural products, particularly sesquiterpene lactones, diterpenes, and flavonoids. They are known to possess wide variety of biological and pharmacological activities such as

antimicrobial, cytotoxic, anti-inflammatory, antiviral, antibacterial, antifungal activities, effects on the central nervous and cardiovascular systems as well as allergenic potency (Chagas-Paula *et al*., 2012). Their wide structural diversity and potential biological activities have made further interest among the chemists (Chaturvedi, 2011).

2.4.2 *Warburgia ugandensis*

Warburgia ugandensis (Canellaceae) is amedicinal plant, traditionally used as herbal medicine for a wide range of diseases in some parts of Ethiopia (Wube *et al*., 2010). The traditional medicinal practitioners use *W. ugandensis* to treat malaria, tuberculosis bronchitis, pneumonia, hepatitis, tapeworm, gonorrhea, and asthma in Dollo Menna, Baleregion of Ethiopia (Wube *et al*., 2010). Most of the biological activities are attributed to the drimane sesquiterpenoids, including polygodial, warburganal, muzigadial, mukaadial and ugandensial, flavonoids and miscellaneous compounds present in the various species (Leonard *et al*., 2015). In addition to anti-infective properties, *Warburgia* extracts are also used to treat a wide range of ailments, including stomach aches, fever and headaches, which may also be a manifestation of infections (Leonard *et al*., 2015). Dried bark is commonly chewed and the juice swallowed as a remedy for stomach-ache, constipation, toothache, cough, fever, muscle pains, weak joints and general body pains (Wamalwa *et al*., 2006; Orwa *et al*., 2009). Fresh roots are boiled and mixed with soup for the prevention of diarrhoea.

2.4.3 *Croton megalocarpus*

Croton megalocarpus belongs to the family Euphorbiaceae consisting of about 7,500 species in 300 genus. The genus *Croton* is pan tropical with some species extending into temperate areas. The leaves, roots and bark are used to treat stomach problems and pneumonia. The seeds have high oil (32%) and protein contents. The bark decoction is used as a remedy for worms and whooping cough. Grounded roots are used for syphilis, anthrax, and snake bites treatment (Kavee, 2013). The water extract of *Croton megalocarpus* has been reported to have activity against bacteria strains (Miaron *et al*., 2014).

2.4.4 *Carissa edulis*

Carissa edulis belonging to Apocynaceae family comprises of about 155-200 genera and 2000 species, distributed primarily in the tropics and subtropics. Plants of Apocynaceae are often poisonous and rich in alkaloids or glycosides especially in the seeds and latex. It has been reported that aqueous total extract preparation from the roots of *Carissa edulis* (Forssk.) Vahl (Apocynaceae), a medicinal plant locally growing in Kenya that has exhibited remarkable anti- HSV activity *in vitro* and *in vivo* for both wild type and resistant strains of HSV. The extract significantly inhibited formation of plaques in Vero E6 cells infected with 100PFU of wild type isolates of HSV (7401H HSV-1 and Ito-1262 HSV-2) or resistant isolates of HSV (TK(-) 7401H HSV-1 and AP(r) 7401H HSV-1) by 100% at 50 µg/ml *in vitro* with minimal cell cytotoxicity (480 mg/ml) (Tolo *et al*., 2006). Preparations of *Carissa edulis* have been used in the Nigerian traditional medicine for the management of fever, sickle cell disease, epilepsy, pain and inflammation for many years and their efficacy is widely acclaimed among the Hausa communities of northern Nigeria (Ya'u *et al*., 2008).

2.4.5 *Launaea cornuta*

Launaea cornuta comprises about 55 species and occurs in Africa and south-western Asia, but a single species (*Launaea intybacea* (Jacq.) Beauverd) has been introduced and naturalized in the Caribbean region and Central America. Northern and eastern Africa is particularly rich in species. *Launaea* is placed in tribe *Lactuceae* subtribe *Sonchinae*, together with e.g. *Reichardia*.

*Launea cornuta (*Asteraceae) is widely used among African communities for its medicinal and nutritional values and a livestock fodder. Traditionally, it is used by Kenyan communities to manage diabetes mellitus, syphilis, and stomach-ache, among many other diseases. Previous studies of phytochemical compounds in the ethyl acetate extracts of *L. cornuta* using gas chromatography coupled to mass spectrometer identified isoquinoline alkaloids, terpenoids and phytosterols, among others. The presence of diverse chemical compounds supports the medicinal and nutritional uses of *L. Cornuta* among African communities (Karau *et al*., 2014).

2.5 Evaluation of antimicrobial plants

Research on medicinal plants as a source of antimicrobial drugs calls for involvement of both pharmacognosists and clinical bacteriologists. The plants are mainly selected on basis of their traditional reputation for efficacy in the treatment of typhoid fever and other diseases. Selected plants are subjected to preparation and/or purification of extracts. Initially it is imperative to go for *in vitro* primary screening which reduces the number of laboratory animals used for experiments. Although agents active *in vitro* are often inactive *in vivo* and vice versa (Muthaura *et al*., 2007), the *In vitro* system can act as a primary screen and helps to identify plants for *in vivo* testing. If a compound kills the bacteria *in vitro*, it will also be screened for toxicity against mammalian cells *in vitro*. A plant's active ingredients are isolated and identified with chromatography, mass spectrometry, nuclear magnetic resonance and other techniques (Colegate & Molyneux, 2007; Rahuman *et al*., 2008). Identified agents are then further tested for their efficacy and toxicity *in vitro*.

In vivo studied are done in animal model, preferably mice infected with bacteria. The activity of the test material *in vivo* is influenced by a number of factors: The compounds effective *in vitro* may not be effective *in vivo* due to their failure to reach the requisite site of action or they are metabolized too quickly to a less active or inactive form. Or a compound can be more active *in vivo* because it gets metabolized into a more active form (Atanasov *et al*., 2015). For example, Berbelis alkaloids have little activity against *Entamoeba histolytica* (Singh, 2011). When a medicinal plant proves effective *in vivo* and shows no host toxicity, its mechanism of killing bacteria is studied through complex and extensive biochemical testing. Also, a likely effect on host's metabolism must be studied before a drug is released for clinical and field trials. For instance, a good drug is that distributes to all potential sites of infection in the body. If toxic, the drug will therefore exert its effects on multiple tissues and body organs. This is especially true for the liver and the kidney, the main sites of metabolism and drug excretion respectively. The idea behind the oral administration of plant extracts to mice is that if the doses prescribed by the traditional practitioners are not toxic to patients, they should not be toxic to mice, under the same conditions, and may even be active. It is

desirable to find out whether a drug has broad spectrum of activity by testing it against a range of like organisms.

2.5.1 Extraction of bioactive compounds from plants

Considering the great variations among bioactive compounds and huge number of plant species, it is necessary to build up a standard and integrated approach to screen out these compounds carrying human health benefits. Azmir *et al*., (2013), reported an integrated approach showing sequence of medicinal plant study, which started from the collection of frequently used plants and ended at industrialization. Works of particular order for medicinal plant study and the position of extraction techniques are well studied areas in research. It is only possible to conduct further separation, identification, and characterization of bioactive compounds followed by an appropriate extraction process. Different extraction techniques should be used in diverse conditions for understanding the extraction selectivity from various natural sources. Different techniques, many of them remain almost same through hundreds of years; can also be used to extract bioactive compounds. All these techniques have some common objectives, (a) to extract targeted bioactive compounds from complex plant sample, (b) to increase selectivity of analytical methods (c) to increase sensitivity of bioassay by increasing the concentration of targeted compounds, (d) to convert the bioactive compounds into a more suitable form for detection and separation, and (e) to provide a strong and reproducible method that is independent of variations in the sample matrix (Viswanathan *et al*., 2007).

Bioactive compounds from plant materials can be extracted by various classical extraction techniques. Most of these techniques are based on the extracting power of different solvents in use and the application of heat and/or mixing. In order to obtain bioactive compounds from plants, the existing classical techniques are: (1) Soxhlet extraction, (2) Maceration and (3) Hydrodistillation (Viswanathan *et al*., 2007).

2.5.2 Column chromatograpy

The methods of separation in chromatography are based on the distribution of the components in a mixture between a fixed (stationary) and a moving (mobile) phase (Braithwaite & Smith, 2012). The stationary phase may be a column of adsorbent, a paper, a thin layer of adsorbent on a glass plate, etc., through which the mobile phase moves on. The mobile phase may be a liquid or a gas. When a solid stationary phase is taken as a column it is known as column chromatography. Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids. This is a solid - liquid technique in which the stationary phase is a solid & mobile phase is a liquid (Braithwaite & Smith, 2012). The principle of column chromatography is based on differential adsorption of substance by the adsorbent. The usual adsorbents employed in column chromatography are silica, alumina, calcium carbonate, calcium phosphate, magnesia, starch, etc., selection of solvent is based on the nature of both the solvent and the adsorbent (Stock & Rice, 2013). The rate at which the components of a mixture are separated depends on the activity of the adsorbent and polarity of the solvent. If the activity of the adsorbent is very high and polarity of the solvent is very low, then the separation is very slow but gives a good separation. On the other hand, if the activity of adsorbent is low and polarity of the solvent is high the separation is rapid but gives only poor separations, i.e., the components separated are not 100% pure. The adsorbent is made into slurry with a suitable liquid and placed in a cylindrical tube that is plugged at the bottom by a piece of glass wool or porous disc. The mixture to be separated is dissolved in a suitable solvent and introduced at the top of the column and is allowed to pass through the column (Stock & Rice, 2013). As the mixture moves down through the column, the components are adsorbed at different regions depending on their ability for adsorption (Stock & Rice, 2013). The component with greater adsorption power will be adsorbed at the top and the other will be adsorbed at the bottom. The different components can be desorbed and collected separately by adding more solvent at the top and this process is known as *elution*.

That is, the process of dissolving out of the components from the adsorbent is called elution and the solvent is called is called eluent. The weakly adsorbed component

will be eluted more rapidly than the other. The different fractions are collected separately. Distillation or evaporation of the solvent from the different fractions gives the pure components (Armarego & Chai, 2013). Intermolecular forces, which vary in strength according to their type, make organic molecules to bind to the stationary phase. The stronger the intermolecular force, the stronger the binding to the stationary phase, therefore the longer the compound takes to go through the column. Intra- molecular hydrogen bonding is present in ortho- nitro phenol. This is due to the polar nature of the oxygen-hydrogen (O-H) bonds which can result in the formation of hydrogen bonds within the same molecule. But in para-nitro phenol, inter molecular hydrogen bonding (between H and O atoms of two different para-nitro phenol molecules) is possible. As result of inter molecular hydrogen bonding para-nitro phenol undergo association that increases the molecular weight, whereby decreasing volatility.

2.6 Dihydrofolate Reductase

Dihydrofolate reductase, DHFR, that role is to regenerate folic acid into its reduced form tetrahydrofolate, is necessary for bacteria, plasmodia and normal cancerous human cells. Inhibitors of DHFR have antibiotic, anti- malarial and anti-neoplastic properties. The mechanism of action according to Nicola *et al*., (2007) showed that, folate and its derivatives are important co-factors in synthesis of nucleotides (DNA) for all organisms (man, Plasmodia, etc.). Synthesizing nucleotides (DNA) required tetrahydrofolate (THF). THF is from Dehydrafolate (DHF) by action of DHFR. Thus, any drug that can selectively inhibit the bacteria DHFR can inhibit and kill the bacteria. Trimethoprim [2, 4-diamino-5-(3, 4, 5-trimethoxybenzyl) pyrimidine] is DHFR inhibitor mainly used as a folic acid antagonist and anti-infecting agent. Its selective bactericidal activity consists on blocking folate synthesis in the bacterial thymidine synthesis pathway, therefore disrupting DNA replication process.

2.6.1 Function and clinical significance of DHFR

Dihydrofolate reductase converts dihydrofolate into tetrahydrofolate, a methyl group shuttle required for the de novo synthesis of purines, thymidylic acid, and certain amino acids (Figure 2.1). While the functional dihydrofolate reductase gene has been mapped to chromosome 5, multiple intronless processed pseudogenes or dihydrofolate reductase-like genes have been identified on separate chromosomes (Schrider *et al.*, 2013).

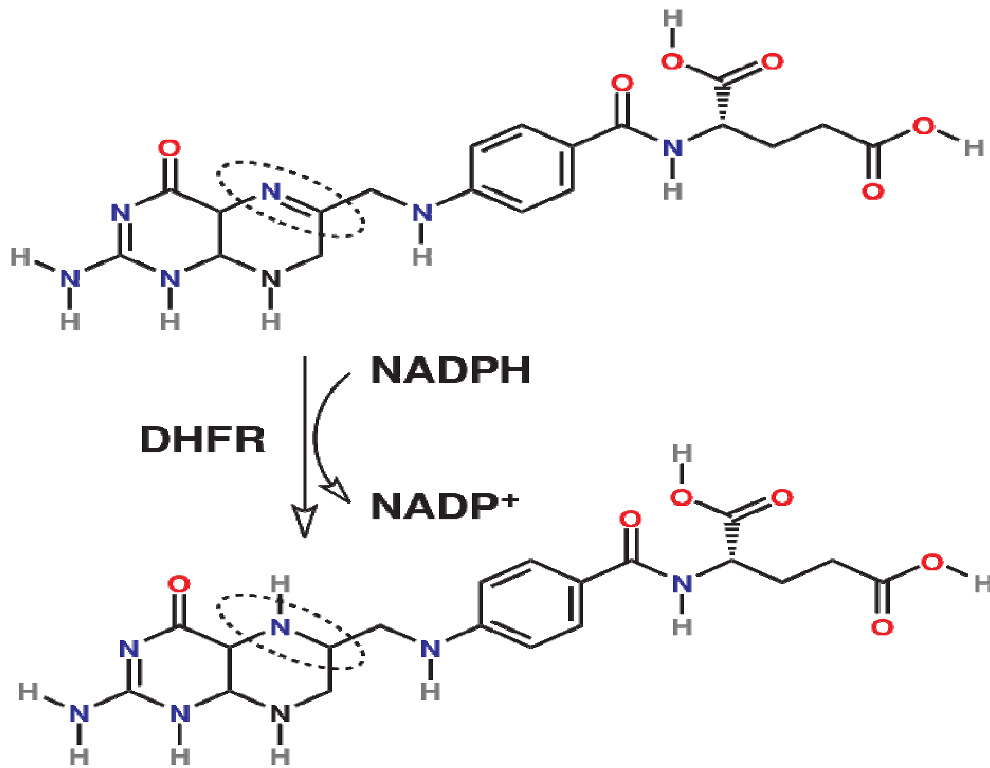


Figure 2.10: Reaction catalyzed by DHFR

Source: (Schrider *et al.*, 2013)

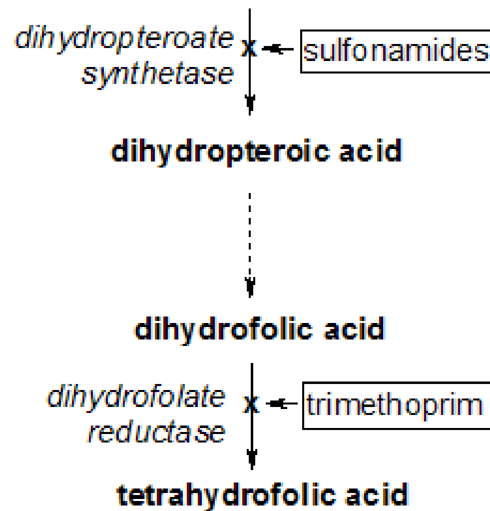


Figure 2.11: Tetrahydrofolate synthesis pathway

Source: (Hawser *et al*., 2006)

Found in all organisms, DHFR has a critical role in regulating the amount of tetrahydrofolate in the cell. Tetrahydrofolate and its derivatives are essential for purine and thymidylate synthesis, which are important for cell proliferation and cell growth (Schnell *et al*., 2004). DHFR plays a central role in the synthesis of nucleic acid precursors, and it has been shown that mutant cells that completely lack DHFR require glycine, an amino acid, and thymidine to grow (Kompis *et al*., 2005). DHFR has also been demonstrated as an enzyme involved in the salvage of tetrahydrobiopterin from dihydrobiopterin (Crabtree *et al*., 2009). DHFR is an attractive pharmaceutical target for inhibition due to its pivotal role in DNA precursor (thymine nucleotides) synthesis. Trimethoprim, an antibiotic, inhibits bacterial DHFR while methotrexate, a chemotherapy agent, inhibits mammalian DHFR.

2.6.2 Mechanism of action of DHFR

DHFR catalyzes the transfer of a hydride from NADPH to dihydrofolate with an accompanying protonation to produce tetrahydrofolate (Schnell *et al*., 2004). In the end, dihydrofolate is reduced to tetrahydrofolate and NADPH is oxidized to NADP+ (Figure 2.3). The high flexibility of Met20 and other loops near the active site play a role in promoting the release of the product, tetrahydrofolate. In particular the Met20 loop helps stabilize the nicotinamide ring of the NADPH to promote the transfer of the hydride from NADPH to dihydrofolate (Arora & Brooks, 2009).

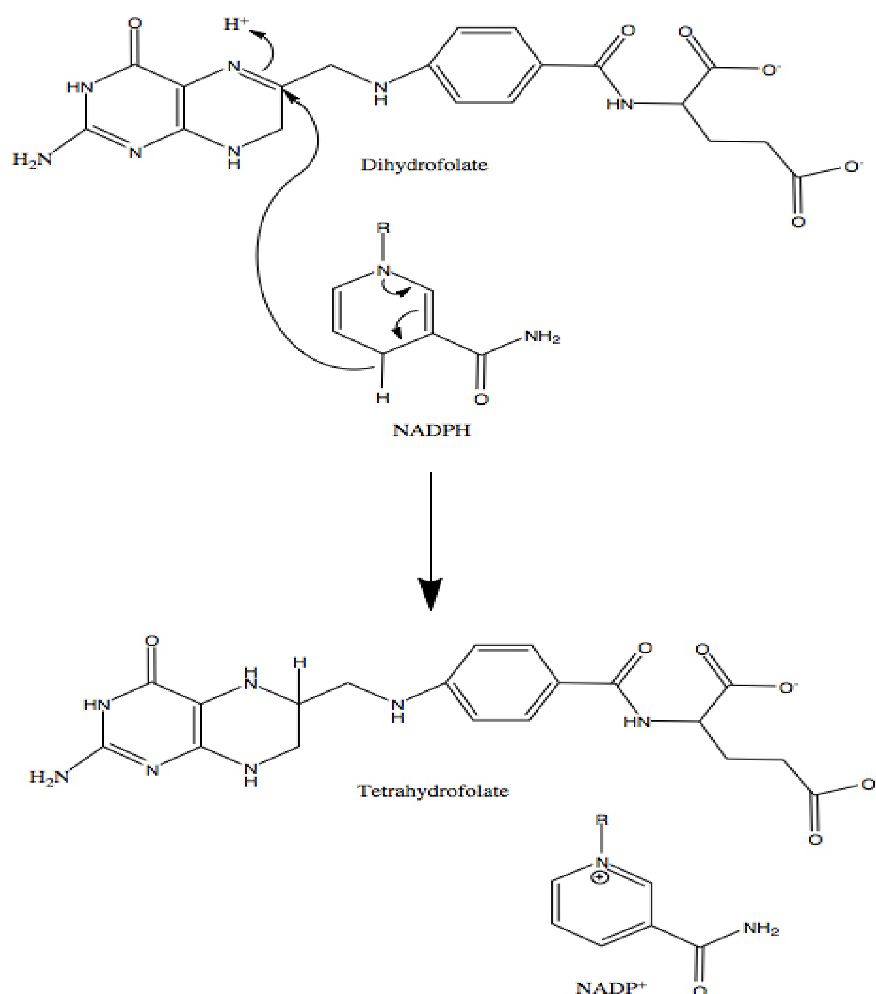


Figure 2.12: The reduction of dihydrofolate to tetrahydrofolate

Source: (Schnell *et al*., 2004)

2.6.3 Therapeutic applications of DHFR

Since folate is needed by rapidly dividing cells to make thymine, this effect may be used to therapeutic advantage. DHFR can be targeted in the treatment of cancer. DHFR is responsible for the levels of tetrahydrofolate in a cell, and the inhibition of DHFR can limit the growth and proliferation of cells that are characteristic of cancer. Methotrexate, a competitive inhibitor of DHFR, is one such anticancer drug that inhibits DHFR (Navarro-Perán *et al*., 2005). Other drugs include trimethoprim and pyrimethamine. These three are widely used as antitumor and antimicrobial agents (Capasso & Supuran, 2014).

Trimethoprim has shown to have activity against a variety of Gram-positive bacterial pathogens (Hawser *et al*., 2006). However, resistance to trimethoprim and other drugs aimed at DHFR can arise due to a variety of mechanisms, limiting the success of their therapeutical uses (Rao & Tapale, 2013). Resistance can arise from DHFR gene amplification, mutations in DHFR, decrease in the uptake of the drugs, among others. Regardless, trimethoprim and sulfamethoxazole in combination has been used as an antibacterial agent for decades (Hawser *et al*., 2006). Folic acid is necessary for growth (Bailey & Ayling, 2009) and the pathway of the metabolism of folic acid is a target in developing treatments for cancer. DHFR is one such target. A regimen of fluorouracil, doxorubicin, and methotrexate was shown to prolong survival in patients with advanced gastric cancer (Boku *et al*., 2009). Further studies into inhibitors of DHFR can lead to more ways to treat cancer.

Bacteria also need DHFR to grow and multiply and hence inhibitors selective for bacterial DHFR have found application as antibacterial agents (Hawser *et al*., 2006). Classes of small-molecules employed as inhibitors of dihydrofolate reductase include diaminoquinazoline and diaminopyrroloquinazoline (Srinivasan & Skolnick, 2015) diaminopyrimidine, diaminopteridine and diaminotriazines (Srinivasan *et al*., 2015).

2.7 Natural products in drug development

According to WHO more than 80% of worlds' population relies on traditional medicine for their primary healthcare, majority of them use plants or their active principles (Sahoo *et al*., 2010; Verma & Singh, 2008). Many plants are used in

Africa continent for treatment of different diseases such as fever, dysentry, Cholera diarrhea etc and others which are typical disease of a tropical country (Ayogu & Amadi, 2009; Ajaji & Akinoti, 2010). Medicinal herbs with unique chemical compounds that can either inhibit the growth of pathogens or kill them and have no or least toxicity to host cells are considered as potential candidates for developing new antimicrobial drugs. Traditional practitioners use herbal preparations to treat microbial infections such as typhoid and paratyphoid infections because they claim that they; are relatively safer than synthetic alternatives, offers profound therapeutic benefits and they are more affordable for treatments. Plants used in this study have traditionally been associated with disease curative and preventive practices in many countries for a long time. Garcia and Delgado (2006) have reported that *T. diversifolia* has promising medicinal value. Skin products formulated from *T. diversifolia* extracts have been shown to have antimicrobial properties (Kareru *et al.*, 2010). In Ethiopia *Warburgia ugandensis* extracts are used to treat malaria, tuberculosis, bronchitis, pneumonia, hepatitis, tapeworm, gonorrhea and asthma (Wube *et al.*, 2010; Were *et al.*, 2010; Opiyo *et al.*, 2011). The decoction from *C. megalocarpus* bark is used as a remedy for worms and whooping cough. Grounded roots are used for syphilis, anthrax, and snakebites treatment (Kabir *et al.*, 2005). Different communities in Africa use parts of *C. edulis* to alleviate pain, treat venereal diseases, glandular inflammation, induce abortion and restore virility (Githiori *et al.*, 2004). In Kenya and Tanzania decoction from *L. cornuta* roots is used as a remedy for cough, typhus fever and measles (Schippers, 2004).

The *T. diversifolia*, *W. ugandensis*, *C. megalocarpus*, *C. edulis* and *L. cornuta* plants were collected in Nyamira County, Kenya, locally reputed to be of remedial value, in order to investigate and verify whether the claimed anti-salmonella properties of these plants by traditional practitioners can be scientifically confirmed and provide support for their use in the treatment and management of typhoid fever. They were evaluated for biological activities of their extracts and fractions using five bioassay protocols, namely, *in vitro* and *in vivo* anti-salmonella potentials, toxicity levels, the ability to inhibit the enzyme DHFR and identification of major compounds by GC-MS technique.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental study site

The *T. diversifolia*, *W. ugandensis*, *C. megalocarpus*, *C. edulis* and *L. cornuta* plants were collected in Nyamira County, Kenya and their extracts were used in this study. Extraction, phytochemical analysis fractionation and identification of bioactive compounds by GC-MS were carried out at Chemistry laboratory, Jomo Kenyatta University of Agriculture and technology (JKUAT). *In vitro* anti-salmonella activity was carried out at CMR-KEMRI laboratory. Acute toxicity, sub- chronic toxicity and histopathology studies were performed at BRI-KALRO. The efficacy and inhibition effects of semi purified fractions against DHFR were carried out at Biochemistry laboratory, JKUAT.

3.2 Experimental design

The parts from five plants (Table 3.1) were processed and phytochemicals extracted using four solvent systems namely; hexane, ethyl acetate, methanol and water. Thirty six extracts obtained were subjected to standard phytochemical analyses as described by Jigna *et al*., (2006). In addition, the extracts were screened for anti-salmonella activity against four clinical isolates. *In vitro* work was done in triplicates and duplicates by disc diffusion and microdilution techniques respectively to confirm anti-salmonella activity. Nine active plant extracts that showed anti-salmonella activity *in vitro* were subjected further for toxicity studies in mice. In acute toxicity, 10 groups of mice (5 per group) were used including the control group and adminstered orally with a single dose of plant extracts (2000mg/kg) . In sub-chronic toxicity, 28 groups mice were used and each plant extract was adminstered orally in three concentrations; 300, 600 and 1200mg/kg for the entire period of experiment. *In vivo* efficacy determination of the nine plant extracts was carried out as follows; 29 groups of mice each group having 5 mice were used which included the positive and negative control groups. Mice were inoculated using gavage needle orally with approximately $1x10^5$ Colony Forming

Units (CFU) of *S. ser. Typhimurium* in 0.2 ml volume and treatment was begun 24 hours post infection with the plant extracts. The active plant extracts were administered orally, for 5 days with the following doses; 300, 600 and 1200mg/kg at v/w ratio 1/100 of mice body weight. All extracts were freshly prepared in 5% of Dimethyl sulfoxide (DMSO). The extracts which showed *in vivo* anti-salmonella activity were fractionated using column chromatography and their fractions tested for anti-salmonella activity and inhibitory effect on DHFR. Compounds in the fractions were identified by GC-MS.

3.3 Clinical *Salmonella* isolates

Clinical samples of *S. ser. Typhi* (ATCC 13347), *S. ser. Typhi* (ATCC 43579), *S. enterica* (ATCC 2162) and *S. ser. Typhimurium* (ATCC 1408), were provided by the Centre of Microbiology Research-Kenya Medical Research Institute (CMR-KEMRI) for this study. They were isolated in 2011 from feacal samples of patients presenting with diarrhea in Mbagathi hospital, Nairobi. The isolates were maintained at -80^0 C in Trypticase broth until use.

3.4 Plant Materials

Five plants (Table 3.1) selected for this study, which are used frequently in treatment of bacterial diseases by traditional practitioners were used. These were collected in October, 2011 in Nyamira County. The plants were authenticated at Jomo Kenyatta University of Agriculture and Technology, Botany Department using standard taxonomical methods as described in appendix (i). The materials were then taken to the laboratory where were carefully washed under running tap water to remove dust and any other foreign material and left to drain off.

Table 3.1: Profile of the five medicinal plants

Botanical name	Common name	Family name	Part of the plant used
T. diversifolia	Tree marigold	Solanaceae	Flowers and leaves
W. ugandesis	Muthiga	Conellaceae	Roots and stem barks
C.megalocarpus	Mukinduri	Euphorbiaceae	Barks
C. edulis	Olamuriaki	Apocynaceae	Roots and barks
L. cornuta	Wild lettuce	Asteraceae	Roots and leaves

3.5 Preparation of plant materials for extraction

The size of the plant materials, roots and stem barks were chopped into small bits by a sharp machete and flowers and leaves were reduced by a knife. They were then spread on laboratory benches and left to air dry for three weeks.

The dried pieces were ground into powder by grinding mill.The weight of each plant material in powder form was determined and their recorded.

3.5.1 Extraction of phytochemicals from plant materials

Five plant materials were extracted using organic and universal solvents. Each plant material was extracted sequentially with hexane, ethyl acetate and methanol in the order of increasing polarity. Single extraction was also carried out using water for each of the five plant materials.

3.5.1.1 Preparation of hexane extract

Approximately 500 g of each plant powder was soaked separately in 1500 ml of hexane into a conical flask with a rubber cork. The contents were kept for 3 days away from direct sunlight, undisturbed, then filtered through sterile filter paper. The filtrate was transferred into sample holder of a rotary vaccum evaporator where the hexane solvent was evaporated at its boiling temperature of 38.5-42 ^{O}C. The standard extract obtained was then weighed recoreded and stored in refrigerator at 4 ^{O}C until required for use. The residued used for ethyl acetetate extraction.

3.5.1.2 Preparation of ethyl acetate extract

The 500g of hexane residues were re-soaked in 1500 ml of ethyl acetate. The contents were kept for 5 days away from direct sunlight, undisturbed and afterward filtered. The filtrate was concentrated at 38.5-42 ^{O}C. The standard extract obtained was then weighed recoreded and stored in refrigerator at 4 ^{O}C until required for use. The residued used for methanol extraction.

3.5.1.3 Preparation of methanol extract

The 500g of ethyl acetate residues were re-soaked in 1500 ml of methanol and kept for 36 hours away from direct sunlight undisturbed. After filtration, the filtrate was concentrated at 65 ^{O}C. The extract obtained was then weighed, recoreded and stored in refrigerator at 4 ^{O}C until required for use. The residued was discarded.

3.5.2 Aqueous extraction

3.5.2.1 Preparation of aqueous extracts

Following the powdered materials from *T.diversifolia*, *W.ugandensis*, *C. megalocarpus*, *C.edulis* and *L. cornuta* were used for aqueous phytochemical extraction. Five hundred grams (500 g) of each of the powdered plant materials was weighed and soaked separately in 1500 ml of distilled water. The contents were warmed in a water bath for 2 hours at 60 ^{O}C, then left to stand at room temperature for 10 hours, undisturbed. They were subsequently filtered off with sterile filter paper (Whatman No. 5) into a clean conical flask and the filtrate was freeze dried to powder, weighed and recorded.

3.5.2.2 Freeze drying process

The products were pre-frozen in appropriate container (i.e. beaker, tray, flask and ampoule); temperature controller was used and the product probe was frozen into the product. The freeze dryer base unit was prepared for operation in accordance with operating instructions. When the condenser temperature reached -40°C, the accessory onto the flange of the base unit was assembled and the rack accessory placed in position over the condenser port. The heater mats were then fitted to the rack by pushing the plug connections into sockets on the spine and the product trays were loaded onto the rack accessory before fitting acrylic chamber.

3.5.3 Determination of phytochemical constituents

The freshly prepared extracts were subjected to standard phytochemical analyses for tannins, alkaloids, terpenoidal saponins, flavanoids, glycosides and steroidal saponins as described by Jigna, *et al*., (2006).

3.5.3.1 Test for tannins

One milliliter of hexane, ethyl acetate, methanol and water extracts will be mixed with 10 mL of distilled water and filtered. Ferric chloride reagent (3 drops) was added to the filtrate. A blue- black or green precipitate confirmed the presence of gallic, tannins or catechol tannins, respectively.

3.5.3.2 Test for alkaloids

A small portion (0.2 mL) of the extract was mixed with 1% aqueous hydrochloric acid (5 mL) and placed on a steam bath. Then 1mL of the content was treated with Mayer's reagent (3 drops) while another portion was be similarly treated with Dragedorff's reagent. Turbidity or precipitation with these reagents indicated presence of alkaloids.

3.5.3.3 Test for steroidal and triterpenoidal saponins

Ten mililitres (10 mls) of plant extract was placed in a small beaker (50 ml) and evaporated to dryness. The residue was redisoived with 0.5 ml of acetic anhydride and 0.5 ml of chloroform. The solution was transferred into dry test tube and then a concentrated solution of sulphuric acid (2 ml) was added slowly. Brownish red, pink or violet rings at the zones of contact with the supernatant and blue or Green colour or a mixture of these two shades denoted the presence of terpenoidal and steroidal saponins respectively.

3.5.3.4 Test for cardiac glycosides

Five (5) ml of each of the extracts was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid. Abrown ring of the interface indicates a deoxysugar characteristic of Cardenolides.

3.5.3.5 Test for flavonoids

Two millilitre filtrate was added to concentrated HCl and magnesium ribbon. The pink-tomato red colour indicated the presence of flavonoids.

3.6 Controls

Water and DMSO were used as negative controls. Ciprofloxacin, lot number; 1006709501 and chloramphenicol, lot number; 1006708575 (Transchem pharmaceutical Ltd, Kenya) were used as positive controls in determining anti-salmonella activity. Trimethoprim, lot number; 1006801568 (positive control) was used as known inhibitor of dihydrofolate reductase (DHFR) enzyme.

3.7 Inhibition assays

3.7.1 Disc diffusion Assay

Disc diffusion method for antimicrobial susceptibility testing was carried out according to the standard method by Bauer *et al*., (1966) to assess the presence of

anti-salmonella activities of the plant extracts. Circular paper discs (6mm diameter) were placed on Muller Hinton media inoculated with clinical *Salmonella* isolates. Sterile paper discs were dampened with 10µl of water and DMSO extracts (1000mg/ml). The loaded disc was placed on the surface of the medium, the compound was allowed to diffuse for 5 minutes and plates were kept for incubation for 24 h at 37 ^{0}C. Antibiotic disc containing ciprofloxacin and chloramphenicol were used as positive controls. A disc loaded with DMSO and water served as negative controls. The anti-salmonella activity was evaluated by measuring the diameter of the inhibition zone formed around the discs. The study was performed in triplicate.

3.8.2 Determination of Minimum Inhibitory Concentration (MIC) values

The MIC values were determined using micro-dilution assay as described by Eloff (1998) and Klančnik *et al*., (2010). Ciprofloxacin and chloramphenicol were used as positive controls and DMSO was used as negative control. Plant extracts were tested against clinical *Salmonella* isolates with varying concentrations ranging from 62.5mg/ml to 0.0305mg/ml. Briefly, 100 µl of sterile distilled water was added to 96-well microtitre plates (SIGMA Aldrich, German) followed by the addition of 100 µl of 62.5mg/ml of the serially diluted plant extracts. Then 100 µl of *Salmonella* isolates were added to each microtitre plates in all the wells to give the final volume of 200 µl. The prepared microtitre plates were sealed to avoid drying and incubated overnight at 37^{0}C. After overnight incubation, 50µl of 5mg/ml 2, 3, 5 Triphenyltetrazolium chloride (SIGMA Aldrich, German) was added to all the microtitre plate wells and incubated overnight again. The pink colour was indicative of bacterial growth while lack or less colour was linked to growth inhibition. The MIC was defined as the lowest concentration of plant extract that completely suppress the growth of clinical *Salmonella* isolates.

3.8 *In vivo* evaluation of toxicity of plant extracts

3.8.1 Evaluation of acute toxicity

Seven weeks old, male Swiss mice weighing between 25-30 g were obtained from BRI-KALRO. Animals were randomly assigned to control and treatment groups (5

mice; per group). They were housed under standard environmental conditions of temperature at 24 + 1 °C under a 12 h dark- light cycle, and allowed free access to drinking water and standard pellet diet (mice cubes). Mice were kept in experimental facility for 1 week to acclimatize prior to dosing. Animals were deprived of food except water 16-18 hour prior to dosing on day 0. According to the World health Organization (WHO) guideline (WHO 2000) and the Organization of Economic Cooperation and Development (OECD) guideline for testing of chemicals 420 (OECD 2001). The extract at the dose of 2,000 mg/kg were given once orally to the test group of mice, while the control groups received water and DMSO in the same volume by gavage using a ball-tipped stainless steel feeding needle (Jaijoy *et al*., 2010). Body weight, Packed Cell volume (PCV), signs of toxicity (general behavior, respiratory pattern and change in skin and fur) and mortality were observed after the administration at the first, second, fourth and sixth hour and once daily for next 14 days (Jaijoy *et al*., 2010). On the 15th day, all mice were kept fasted overnight, and later anesthetized with thiopental sodium (50 mg/kg). Mice were sacrificed for histopathology studies (Liver and Kidney were removed for further analysis). Histological examination of the tissues was performed to confirm pathology.

3.8.2 Evaluation of sub-chronic toxicity

Seven weeks old, Swiss mice (25-30g) were randomly divided into groups (5 mice per group). The extracts were administered orally at concentrations of 300, 600 and 1,200 mg/kg to treatment groups for consecutive 28 days, twice daily, while the control group received DMSO. The animals were monitored for 30 days (Jaijoy *et al*., 2010).

General behavior, respiratory pattern, change in skin and fur, mortality and the body weight changes were monitored daily as described by Jaijoy *et al*., 2010. At the end of the study, they were kept fasted for 16-18 h and then anesthetized with intraperitoneal injection of thiopental sodium at a dose of 50 mg/kg on the 31st. All mice were sacrificed and liver and kidney removed for histopathological studies (BRI-KALRO).

3.8.3 Tissue processing

Pieces of tissue samples from the right lobe of liver and the right kidney (Section 3.8 subsections 3.8.1 and 3.8.2) were immersed in 10% buffered neutral formalin (pH=7.0) overnight at room temperature. After overnight fixation, the tissue samples were washed for 6–8 hours in tap water then after, the tissue samples were dehydrated with graded series of alcohol: one hour each in 70% alcohol, 80% alcohol, 95% alcohol and absolute alcohol-I, and two hours in absolute alcohol-II. The tissues were cleared with two changes of xylene, one hour each. The tissues were then infiltrated with two changes of paraffin wax, for one and half hour each. Upon completion of infiltration, the tissues were embedded in the paraffin wax. All tissue blocks were labeled and placed in refrigerator until sectioned.

Tissue blocks were sectioned with a thickness of 5μm using Leica rotary microtome. After the sections were appropriately spread on a water bath, they were mounted on slides coated with egg albumin to maximize surface adhesion. The slides were arranged in slide racks and were placed in an oven with a temperature of 60°C for 10–15 minutes. The tissue sections were then cooled dried and stained with routine Hematoxylin and Eosin staining method. The histopathological slides were then read using light microscope, magnification x400. The experiment was carried out from BRI-KALRO.

3.8.4 Determination of PCV

This is a fraction of whole blood volume that consists of red blood cells. The blood was obtained by bleeding tail vein of mice. The blood was collected in heparinized capillary tubes which was sealed immediately. The capillary tubes with the blood were then centrifuged in a micro-centrifuge for 5 minutes at 10000 *g* rpm. After centrifugation, the height of the red blood cell column was measured by use of haematocrit reader and compared to the total height of the column of the whole blood. The percentage of the total blood volume occupied by red blood cell mass is the haematocrit which depends mostly on the number of red blood cells. The reference values are 42-52% for males and 36-48% for females. The haematocrit is usually about 3 times haemoglobin value (assuming there is no marked

hypochronia). The average error in haemotocrit is about 1-2%. The haematocrit may be changed by altitude and position as in the case of haemoglobin. So, descrease in PCV against the reference indicates the presence of infection and/or vital organs/cells are raptured.

3.9 Determination of *in vivo* efficacy of the plant extracts

3.9.1 *In vivo* experimental design

The animal ethics committee of JKUAT approved(Appendix ii) *in vivo* efficacy protocols before using them, according to the World Health Organization (WHO) guideline (WHO 2000) and the Organization of Economic Cooperation and Development (OECD) guideline for testing of chemicals 420 (OECD 2001). Five mice were allocated to each group and 9 plant extracts were evaluated in three concentrations; 300, 600 and 1,200 mg/kg post infection, twice daily for five days (Jaijoy *et al*., 2010). One group was infected and the second group infected and treated with DMSO which served as the negative control. The third group was infected and treated with the reference drug ciprofloxacin (For oral administration, the ciprofloxacin was dissolved in sterile water and diluted in water containing 5% glucose). Through the experiment, mice were provided with water containing streptomycin (5mg/ml) in order to reduce the level of facultative anaerobic bacteria that normally colonize the mouse intestine (Myhal *et al*., 1982). The inhibition of the growth of the test organisms were determined by monitoring *S. ser. Typhimurium* in the feces of mice.

3.9.2 *In vivo* anti-salmonella activity of the plant extracts

The efficacy testing was carried out as described by Lee *et al*., 2006. Briefly, *S. ser. Typhimurium* were grown overnight in Luria-Bertani broth (Difco), centrifuged, washed in phosphate-buffered saline (PBS) and then diluted into 20% sucrose to achieve a final concentration of $1x10^5$CFU. The mice were inoculated using gavage needle orally with approximately $1x10^5$CFU of *S. ser. Typhimurium* in 0.2 ml volume. One day after infection animals in the test groups were orally administered with the plant extracts using gavage needle twice daily, whereas the control mice

were not. Fecal samples from each mice cage were collected 0, 1, 2, 3, 4, 5 and 6 days after the bacterial suspensions were administered. Thereafter weekly for 30 days post treatment and the numbers of the bacteria per gram of faeces were determined and also weight of mice were recorded thrice a week for 30 days. Aliquots (100µl) of fecal suspensions were serially diluted in PBS and then plated on duplicate *Salmonella-shigella* agar plates (Difco), which were subsequently incubated overnight at 37°C. Typical colonies were counted on plates that contained between 30 and 300 colonies (Lee *et al*., 2006). The changes in body weight, bacteremia levels, and number of animals survived/death were determined and recorded.

3.10 Fractionation of plant extracts by column chromatography

Methanol extract of *T. diversifolia* leaf, ethyl acetate and hexane extracts of stem bark and root respectively of *W. ugandensis* that were observed to have anti-salmonella activity in mice were subjected to fractionation using column chromatography.

A 60 cm long glass column with the diameter of 10 cm was filled with 1.5 kg of silica gel, mesh size 60-120. Methanol extracts of *T. diversifolia* leaf, ethyl acetate extracts of *W. ugandensis* bark and hexane extracts of *W. ugandensis* root were subjected separately to column chromatography. About 20 gm of methanol extracts were mixed with 200gm of silica gel. The column was eluted with mobile phase of hexane followed by hexane- ethyl acetate with increasing polarity. Fifty fractions of 50ml each were collected, analyzed by Thin Layer Chromatography (Appendix v), TLC, (Merck, S 0,032-0,063mm) using solvent mixture of dichloromethane, chloroform and ethyl acetate in the ratio of 3:2:1 and those with same Retention factor (R_f) were pooled to give 7 column chromatography fractions. For the ethyl acetate and hexane extracts were fractionated following the same procedure and 20 and 100 fractions were collected respectively. They were pooled after TLC run to give 4 and 7 column chromatography fractions of ethyl acetate and hexane extracts respectively. All the obtained column chromatography fractions were collected in sample vials and stored at 20°C until required for use.

3.11 Determination of MIC values of column chromatographyfractions

The MIC values were determined using micro dilution assay as described by Eloff (1998) and Klančnik *et al*., (2010). Ciprofloxacin was used as positive controls and acetone was used as negative control. Plant fractions were tested against clinical *Salmonella* isolates with varying concentration ranging from 2.5mg/ml-0.0012mg/ml. Briefly, 100 µl of sterile distilled water was added to each well of 96-well microtitre plates (SIGMA Aldrich, German) followed by the addition of 100 µl of 2.5mg/ml and thereafter serially diluted plant fractions. Then 100 µl of clinical *Salmonella* isolates were added to each micro well to give a final volume of 200. The prepared plates were sealed to avoid drying and incubated overnight at 37^0C. After overnight incubation, 50µl of 5mg/ml 2, 3, 5 Triphenyltetrazolium chloride (SIGMA Aldrich, German) was added to the wells and incubated overnight. The pink colour was indicative of bacterial growth while lack of color was linked to growth inhibition. The MIC was defined as the lowest concentration of plant fraction that completely suppresses the growth of clinical *Salmonella* isolates.

3.12 Phytochemical analysis of bioactive column chromatography fractions

The freshly prepared column chromatography(CC) fractions were subjected to standard phytochemical analyses for tannins, alkaloids, terpenoidal saponins, flavanoids, glycosides and steroidal saponins as described by Jigna *et al*., 2006, (Section 3.5.3).

3.13 Effect of CC fractions on dihydrofolate reductase Activity

3.13.1 Extraction of cytoplasmic DHFR from *S. ser. Typhimurium* (ATCC 1408)

Extraction of cytoplasmic DHFR from *S. ser. Typhimurium* (ATCC 1408) was done according to Sandrini *et al*., (2014) protocol. The *S. ser. Typhimurium* (ATCC 1408) was cultured in nutrition agar broth and the expected cultures were in logarithmic or stationary phase. The *S. ser. Typhimurium* were harvested by centrifugation at 6,708 x *g* for 10 minutes at 4°C and the culture supernatant was discarded. Pellet was washed by re- suspending it uniformly with Tris base (pH

7.5) to a volume at 50 times that of the pellet. This was followed with re-centrifugation at 6,708 x *g* for 10 minutes and the wash step was repeated. The separation of *S. ser. Typhimurium* membrane proteins, the pellet was washed in 10mM Tris base (pH 7.5). Culture suspension was frozen overnight to weaken the cell wall and increase the lysis of *S. ser. Typhimurium* cells by sonication. *Salmonella ser.Typhimurium* suspension was thawed on ice and placed in a non-breakable plastic beaker sitting in an ice water bath. The sonicator was set between 6- 8 microns and cells were lysed in cycles of 15 seconds sonication followed by 45 seconds cooling until the lysate changed from an opaque solution into a less turbid solution. The lysate sample was centrifuged at 6,708 x *g* for 10 minutes at 4°C to remove large debris fragements and unlysed cells (contained in the pellet). The supernatant containing the total protein extract (membrane and cytoplasmic) was transferred into appropriate centrifuge tubes. The supernatant was re-centrifuge at 10,000 x *g* for 10 minutes at 4°C to separate cytoplasmic proteins from membrane proteins. After centrifugation the supernatant contained the cytoplasmic proteins and pellet contained membrane proteins (was discarded because it did not contain the protein of interest; dihydrofolate reductase enzyme). The concentration of cytoplasmic protein was measured using Biuret protein assay via a spectrophotometer set at 550nm.

3.13.2 Enzymatic assay of dehydrofolate reductase

The DHFR assay was carried out at Biochemistry laboratory, College of Health Sciences, JKUAT, Kenya. DHFR extracted from *S. ser.Typhimurium* (ATCC 1408) was used. Reagents used were, 50mM Potassium phosphate buffer (pH 6.5 at 25ºC), 0.11mM ß-NADPH, 2.3mM Dihydrofolate solution, 0.1 %(w/v) Bovine Serum Albumin (BSA) and DHFR extract. The detailed procedure is as described in appendix (iii).

Spectrophotometer was set at 340 nm and 25 °C, kinetic program (reading every 0.5 minutes for 5 minutes.

Assay reagents were added according to the setup shown in Table 3.2.

Table 3.2: Setup for DHFR activity and DHFR activity inhibition

Tube No.	ß-NADPH (0.11mM)	DHF (2.3mM)	DHFR	H2O	TMP 512mg/ml	KH_2PO_4 Buffer	Plant fractions(mg/ml)		
							128	256	512
1	3.1ml	0.1ml	------	-----	-----	0.1ml			
2	3.1ml	0.1ml		0.1ml					
3	3.0 ml	0.1 ml	0.1ml	----	-----	----			
4	2.9ml	0.1ml	0.1ml		0.1ml				
5	2.9ml	0.1ml	0.1ml				0.1ml		
6	2.9ml	0.1ml	0.1ml					0.1ml	
7	2.9ml	0.1ml	0.1ml						0.1ml

Tube1=Blank (Potassium phosphate buffer), Tube2=Blank (Water), Tube3=DHFR activity, Tube 4=effect of TMP on DHFR, Tube 5, 6 and 7 =the effect of plant fractions on DHFR.

To determine the effect of the plant fractions on DHFR, 0.1ml of fraction was added into suitable quartz cuvette containing 2.9ml of 0.11mM NADPH solution and 0.1ml of 2.3mM dihydrofolic acid solution. The cuvette was covered using Parafilm and mixed by inversion and 0.1ml of DHFR was added just before starting the reaction. After inversion immediately, the cuvette was inserted into the spectrophotometer. The kinetics program was started immediately at 340 nm. Tests were performed in triplicates and the mean value was used to calculate the inhibitory effect of plant extracts.

DHFR activity was calculate by the formula:

$$\text{Units/ml enzyme} = \frac{(\Delta A_{340nm}/\min \text{Test} - \Delta A_{340nm}/\min \text{Blank})(3.2)(df)}{(6.22)(0.1)}$$

Specific activity was also calculated by the formula:

$$\text{Units/mg P} = \frac{\text{Units/ml enzyme}}{\text{Mg proptein/ml enzyme}}$$

Units/mg P: Specific activity in µmol/min/mg protein

3.2=Total volume (in milliliters) of assay

df= Dilution factor (5)

6.22= Extinction coefficient of NADPH at 340nm ($mM^{-1}cm^{-1}$)

0.1 =Volume (in millilitres) of assay

Effect of plant fractions on DHFR activity was expressed as percentage and calculated by the formula:

% inhibition= (1-A) x100,

$$\text{Where } A = \frac{\text{Specific activity of the enzyme with plant fraction}}{\text{Specific activity of the enzyme without plant fraction}}$$

3.14 Gas Chromatography-Mass Spectra

Gas Chromatography-Mass Spectra (GC-MS) analysis was performed at Jomo Kenyatta University of Agriculture and Technology, Juja, Kenya. The methanol, ethyl acetate and hexane fractions (in paste form) were constituted in 2 ml of methanol, ethyl acetate and hexane respectively. The fractions were then subjected to GC-MS analysis. Chromatographic separation was carried out with CE GC 8000 top MSMD 8000 Fyson instrument with Db 35 mr column (10 m x 0.5 mm, 0.25 mm film thickness). Heating programs were executed from 100-250ºC at 3 minutes by using helium as a carrier gas with a flow rate of 1ml/min in the split mode (1:50). An aliquot (2 ml) of oil was injected into the column with the injector heater at 250ºC. Analytical conditions Injection temperature at 250ºC, interface temperature at 200ºC, quadruple temperature at 150ºC and ion source temperature at 230ºC were maintained. Injection was performed in split less mode.

3.15 Identification of compounds in plant fractions

The mass spectra of compounds in samples were obtained by electron ionization (El) at 70 eV, and the detector operated in scan mode from 20 to 600 atomic mass units (amu). Identification was based on the molecular structure, molecular mass and calculated fragments. Interpretation on mass spectrum GC-MS was conducted using the data base of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The name, molecular weight and structure of the components of the test materials were ascertained. The spectrum of the unknown component was compared with the spectrum of the component stored in the NIST library version (2005), software, Turbomas 5.2.

3.16 Statistical Analysis

To assess the effect of plant extracts on clinical *Salmonella* isolates, biological data of treated and control animals were expressed in Mean±SD and statistically analyzed by one way analysis of variance (ANOVA) using using Statistical Analysis Software (SAS, Version 9.0). Difference in values at $P<0.0001$ was considered as statistically significant. Summary of data analysed is described in appendix (viii).

CHAPTER FOUR

RESULTS

4.1 Yields of plant extracts

After drying of leaves and flowers of *T. diversifolia*, stem barks and roots of *W. ugandensis*, roots and leaves of *L. cornuta*, roots and barks of *C. edulis* and barks of *C. megalorcarpus*, 800 g of material was sieved and 500 g was used for extraction. The percentage of yield were calculated by using the formula (IARC, 2002).

$$\text{Percentage of yield}(\%) = \frac{\text{Amount of extract yield}(g)}{\text{Amount of dried plant extracted}(g)} x100$$

The highest yield was (8.6%) for methanol extract followed by hexane extract (8.1%), while the aqueous extract was (0.79%) of *C. edulis* root respectively. Meanwhile the lowest yield was (0.62%) for hexane extract of *L.cornuta* root. The yields and percentage yields of the plant extracts using four solvents is shown in Table 4.1.

Table 4.1: Yields and percentage yields of plant extracts

Plants parts	Hexane extract		Ethyl acetate extract		Methanol extract		Aqueous extract	
	yield	% yield	Yield	% yield	yield	% yield	yield	% yield
WUR	19.5 g	3.9	27.6 g	5.52	25.6 g	5.12	4.54 g	0.91
WUSB	10.2 g	2.04	13.7 g	2.74	33.6 g	6.72	6.2 g	1.24
TDF	18.6 g	3.72	14.9 g	2.98	17.15 g	3.43	8.2 g	1.67
TDL	25 g	5	18.6 g	3.72	23.11 g	4.62	12.4g	2.48
LCR	3.1g	0.62	5.5g	1.1	16.5 g	3.3	28.68 g	5.74
LCL	6.7 g	1.34	13 g	2.6	11.8 g	2.36	3.38 g	0.68
CER	40.5 g	8.1	19.2 g	3.84	43 g	8.6	3.96 g	0.79
CEB	5.7 g	1.14	3.1 g	0.62	8.8 g	1.76	3.05 g	0.61
CMB	20.9 g	4.18	17.6 g	3.52	19.23 g	3.85	10.3 g	2.06

Key: WUR= *W. ugandensis* roots, WUSB= *W. ugandensis* stem bark, TDF= *T. diversifolia* flowers, TDL= *T. diversifolia* leaves, LCR= *L. cornuta* roots, LCL= *L. cornuta* leaves, CER= *C. edulis* roots, CEB= *C. edulis* barks and CMB= *C. megalorcarpus* barks.

4.2 Phytochemical analysis of plant extracts

Phytochemical analysis of the 36 plant extracts revealed the presence of medicinally active constituents in leaf, stem bark, root and flower of the five selected medicinal plants. The analysis demonstrated the presence of alkaloids, tannins, flavanoids, steroidal saponins, terpenoidal saponins and glycosides in the plant extracts (Table 4.2). Steroidal saponins, flavonoids and terpenoidal saponins were detected albeit at varying levels in all the plant extracts. Alkaloids were also found in all methanol, ethyl acetate, aqueous except hexane extracts of the five plants (Table 4.2). Glycosides and taninns and were also detected in different concentration in plant extracts analysed (Table 4.2).

Table 4.2: Phytochemical constituents of the active plant extracts

Plant extracts	Alkaloids	Tanins	Flavanoids	Steroidal-saponins	Terpenoidal-saponins	Glycosides
TDLA	+++	+	+	+	++	++
TDLH	+	++	+	+++	+	+
TDLE	+	+	+	+++	+	+
TDLM	+	++	+	+++	+	-
TDFA	+++	+	+	+	+++	++
TDFH	-	-	-	+++	+	-
TDFE	+	+	+	+++	+	-
TDFM	+	+++	+	+	++	+
WURA	+++	+	+	+	+++	+
WURH	-	+	+	++	++	-
WURE	+	+	+	++	++	-
WURM	+++	+	+	+	+	+
WUSBA	+++	++	+	+	+++	+++
WUSBH	-	-	+	++	++	-
WUSBE	+	+	+	++	+	++
WUSBM	+++	+++	+	+	+++	+++
LCRA	+++	+	+	+	+++	++
LCRH	-	-	+	+	+	-
LCRE	++	+	+	+	+	+
LCRM	+++	+	+	+	+	+
LCBA	+++	+	+	+	++	+
LCBH	-	-	+	+	+	-
LCBE	+	+	+	+	+	+
LCBM	+++	+	+	+	++	+
CERA	+++	+	+	+	+	+
CERH	-	-	+	+	+	-
CERE	+	+	+	+	+	+
CERM	+++	+++	+	+	++	++
CEBA	+++	+	+	+	++	+
CEBH	-	-	+	+	+	-
CEBE	+	+	+	+	+	+
CEBM	+++	+++	+	+	+++	++
CMBA	+++	+	+	+	++	+
CMBH	-	-	+	+	+	-
CMBE	+	+	+	+	+	+
CMBM	++	+++	+	+	+	+

Key: - =Absent; += Present; ++= Moderate concentration; +++= High concentration, TDLA= *Tithonia diversifolia* leaf extract of aqueous, TDLH= *Tithonia diversifolia* leaf extract of hexane, TDLE= *Tithonia diversifolia* leaf extract of ethyl acetate, TDLM= *Tithonia diversifolia* leaf extract of methanol, TDFA= *Tithonia diversifolia* flower extract of aqueous, TDFH= *Tithonia diversifolia* flower extract of hexane, TDFE= *Tithonia diversifolia* flower extract of ethyl acetate, TDFM= *Tithonia diversifolia* flower extract of methanol, WURA= *Warburgia ugandensis* root extract of aqueous, WURH= *Warburgia ugandensis* root extract of hexane, WURE= *Warburgia ugandensis* root extract of ethyl acetate, WURM= *Warburgia ugandensis* root extract of methanol,

WUSBA=*Warburgia ugandensis* stem bark extract of aqueous, WUSBH= *Warburgia ugandensis* stem bark extract of hexane WUSBE= *Warburgia ugandensis* stem bark extract of ethyl acetate, WUSBM= *Warburgia ugandensis* stem bark extract of methanol, LCRA= *L. cornuta* root extract of aqueous, LCRH= *L. cornuta* root extract of hexane, LCRE= *L. cornuta* root extract of ethyl acetate, LCRM= *L. cornuta* root extract of methanol, LCBA= *L. cornuta* bark extract of aqueous, LCBH= *L. cornuta* bark extract of hexane, LCBE= *L. cornuta* bark extract of ethyl acetate, LCBM= *L. cornuta* bark extract of methanol, CERA= *C. edulis* root extract of aqueous, CERH= *C. edulis* root extract of hexane, CERE= *C. edulis* root extract of ethyl acetate, CERM= *C. edulis* root extract of methanol, CEBA= *C. edulis* bark extract of aqueous, CEBH= *C. edulis* bark extract of hexane, CEBE= *C. edulis* bark extract of ethyl acetate, CEBM= *C. edulis* bark extract of methanol, CMBA= *Croton megalocarpu*s bark extract of aqueous, CMBH= *Croton megalocarpu*s bark extract of hexane, CMBE= *Croton megalocarpu*s bark extract of ethyl acetate and CMBM= *Croton megalocarpu*s bark extract of methanol.

4.3 *In vitro* evaluation of anti-salmonella activity of selected plant extracts

4.3.1 Zones of inhibition of plant extracts

Out of 36 plant extracts, nine were found to inhibit the growth of clinical *Salmonella* organisms at 1000mg/ml (Table 4.3). The hexane (flowers), ethyl acetate and methanol (leaves) extracts of *T. diversifolia* were active against *S. ser. Typhi* (ATCC 13347), *S. ser .Typhi* (ATCC 43579), *S. enterica* (ATCC 2162) and *S. ser. Typhimurium* (ATCC 1408). Hexane extract of leaf and ethyl acetate extract of flower from *T. diversifolia* inhibited growth of *S. ser. Typhi* ATCC (13347). The hexane and ethyl acetate extracts of root and stem bark from *W. ugandensis* were also active in stopping the growth of the selected clinical *Salmonella* isolates. Ciprofloxacin and Chloramphenicol had activity against all the clinical *Salmonella* isolates (Table 4.3). The anti-salmonella activity of the nine plant extracts was evaluated using Disc diffusion technique. Ethyl acetate extract of *T. diversifolia* flower inhibited *S. ser.Typhi* (ATCC 43579) and *S. ser. Typhi* (ATCC 13347) with 18.5 ±0 mm and 18±2 mm respectively. Hexane extract of *T. diversifolia* leaf inhibited the above said clinical isolates at 17.±0 mm and 17.67±2 mm respectively (Table 4.3). The active extracts from *W. ugandensis* inhibited all clinical *Salmonella* isolates in the range of 6±0 to 8.67±0.58 and 6.33±0.58 to14±1 mm respectively. Methanol extracts of *T. diversifolia* leaf inhibited all the clinical isolates at 8-12mm (Table 4.3). Twenty seven plant extracts had no anti-salmonella activity and compared well with DMSO (6±0 mm) as shown in Table 4.3.

Table 4.3: Zones of inhibition (mm) of different extracts of selected medicinal plants

Plant extracts/Controls	Mean diameter of inhibition zones (mm)			
	S.ser.Typhi **ATCC 13347**	***S.ser.Typhi*** **ATCC 43579**	***S.enterica*** **ATCC 2162**	***S.ser. Typhimurium*** **ATCC 1408**
TDLA	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
TDLH	17.67±2.08^{f}	17±0^{f}	6±0.00^{l}	6±0.00^{l}
TDLE	10±0^{hij}	10±0.58^{hij}	7.33±0.58^{kl}	7.33±2.31^{kl}
TDLM	11±1^{h}	11.5±58h	7.33±4.0.58^{kl}	11.67±0.58^{h}
TDFA	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
TDFH	15.67±2.08^{g}	15.75±1.15^{g}	7.33±1.15^{kl}	6±0.00^{l}
TDFE	18±2^{f}	18.5±0^{f}	6±0.00^{l}	6.67±0.58^{kl}
TDFM	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
WURA	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
WURH	14±1^{g}	8.33±0.58^{jkl}	6.67±1.15^{kl}	10.67±4.62^{hi}
WURE	8.67±0.58^{ijk}	6±0.00^{l}	6.67±0.58^{kl}	6.67±1.15^{kl}
WURM	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
WUSBA	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
WUSBH	11±3.21^{h}	7.33±2.31^{kl}	6.33±0.58^{kl}	7.33±0.58^{kl}
WUSBE	6±0.00^{l}	**7±0**^{kl}	6.33±0.58^{kl}	6±0.00^{l}
WUSBM	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
LCRA	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
LCRH	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
LCRE	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
LCRM	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
LCBA	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
LCBH	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
LCBE	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
LCBM	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
CERA	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
CERH	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
CERE	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
CERM	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
CEBA	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
CEBH	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
CEBE	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
CEBM	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
CMBA	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
CMBH	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
CMBE	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
CMBM	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
DMSO(-)	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
Water	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}	6±0.00^{l}
CHLO(+)	23.33±0.58^{de}	24±1.73^{cde}	24.33±0.58^{cde}	8.67±0.58^{jk}
CIPRO(+)	26±2^{abc}	23.33±2.52^{a}	26±0^{abc}	19.67±1.53^{f}

Key: IZ=Inhibition zone (in mm) includes the diameter of the disc, , TDLA= *Tithonia diversifolia* leaf extract of aqueous, TDLH= *Tithonia diversifolia* leaf extract of hexane, TDLE= *Tithonia diversifolia* leaf extract of ethyl acetate, TDLM= *Tithonia diversifolia* leaf extract of methanol, TDFA= *Tithonia diversifolia* flower extract of aqueous, TDFH= *Tithonia diversifolia* flower extract

of hexane, TDFE= *Tithonia diversifolia*flower extract of ethyl acetate, TDFM= *Tithonia diversifolia* flower extract of methanol,WURA= *Warburgia ugandensis* root extract of aqueous, WURH= *Warburgia ugandensis* root extract of hexane, WURE= *Warburgia ugandensis* root extract of ethyl acetate, WURM=*Warburgia ugandensis* root extract of methanol, WUSBA=*Warburgia* ugandensis stem bark extract of aqueous, WUSBH= *Warburgia ugandensis*stem bark extract of hexane WUSBE= *Warburgia ugandensis*stem bark extract of ethyl acetate, WUSBM= *Warburgia ugandensis* stem bark extract of methanol, LCRA= *L. cornuta* root extract of aqueous, LCRH= *L. cornuta* root extract of hexane, LCRE= *L. cornuta* root extract of ethyl acetate, LCRM= *L. cornuta* root extract of methanol, LCBA= *L. cornuta* bark extract of aqueous, LCBH= *L. cornuta* bark extract of hexane, LCBE= *L. cornuta* bark extract of ethyl acetate, LCBM= *L. cornuta* bark extract of methanol,CERA= *C. edulis* root extract of aqueous, CERH= *C. edulis* root extract of hexane, CERE= *C. edulis* root extract of ethyl acetate, CERM= *C. edulis* root extract of methanol, CEBA= *C. edulis* bark extract of aqueous, CEBH= *C. edulis* bark extract of hexane, CEBE= *C. edulis* bark extract of ethyl acetate, CEBM= *C. edulis* bark extract of methanol, CMBA= *Croton megalocarpu*s bark extract of aqueous, CMBH= *Croton megalocarpu*s bark extract of hexane, CMBE= *Croton megalocarpu*s bark extract of ethyl acetate and CMBM= *Croton megalocarpu*s bark extract of methanol. Value of $6±0^{l}$=Represents no activity. DMSO (-) =Dimethyl sulphur dioxide (Negative control), CIPRO (+) = Ciprofloxacin (Positive control), CHLO (+) = Chloramphenicol (Positive control). Values are means of triplicate readings (Means±SD). Means followed by different superscript letters in the table above are significantly different at P<0.0001.

4.3.2 Minimum Inhibitory Concentration (MIC) values of plant extracts

The MIC values of nine active plant extracts was evaluated and shown to range from 0.031 to 15.63 mg/ml. The hexane extracts of *T. diversifolia* leaf and flower had an MIC of 0.24-1.95 and 0.12-3.91 respectively. The hexane extracts of *W. ugandensis* root and bark had MIC values of 0.031- 3.91mg/ml and 0.031-0.488 mg/ml respectively. Table 4.4 shows relative potency of the nine active plant extracts compared with reference drugs; ciprofloxacin and chloramphenicol. The the realative potence of the nine active plant extracts compared well with those of standards in anti-salmonella activity. Extracts from *W. ugandensis* were the most active against clinical *Salmonella* isolates tested (Table 4.4).

Table 4.4: Minimum Inhibitory Concentration (mg/ml) of hexane, ethyl acetate and methanol extracts

Plant extracts	***Salmonella* organisms**			
	S.ser.Typhi **ATCC 13347 mg/ml**	***S.ser.Typhi*** **ATCC 43579 mg/ml**	***S.enterica*** **ATCC 2162 mg/ml**	***S.ser. Typhimurium*** **ATCC 1408 mg/ml**
TDLE	0.24^{f}	0.061^{h}	0.031^{j}	0.98^{d}
TDFH	0.98^{d}	0.12^{g}	3.91^{b}	3.91^{b}
TDLM	0.24^{f}	0.031^{j}	0.98^{d}	0.488^{e}
TDLH	0.24^{f}	0.24^{f}	1.95^{c}	0.488^{e}
TDFE	0.98^{d}	15.63^{a}	0.12^{g}	3.91^{b}
WURE	0.24^{f}	0.031^{j}	0.061^{h}	0.12^{g}
WURH	0.031^{j}	0.031^{j}	3.91^{b}	0.031^{j}
WUSBE	0.031^{j}	0.031^{j}	0.061^{h}	0.031^{j}
WUSBH	0.031^{j}	0.031^{j}	0.488^{e}	0.0467^{i}
DMSO(-)	NA	NA	NA	NA
CHLO(+)	0.022^{klm}	0.029^{jk}	0.024^{ijk}	0.030^{j}
CIPRO(+)	0.02^{lm}	0.015^{m}	0.018^{lm}	0.025^{ijk}

Key: NA =No activity, TDLE=*Tithonia diversifolia* leaf extract of ethyl acetate, TDFH= *Tithonia diversifolia* flower extract of hexane, TDLM= *Tithonia diversifolia* leaf extract of methanol, TDLH= *Tithonia diversifolia* leaf extract of hexane, TDFE= *Tithonia diversifolia* flower extract of ethyl acetate, WURE=*Warburgia ugandensis* root extract of ethyl acetate, WURH= *Warburgia ugandensis* root extract of hexane, WUSBE= *Warburgia ugandensis* stem bark extract of ethyl acetate, WUSBH= *Warburgia ugandensis* stem bark extract of hexane, DMSO(-VE)= Dimethyl sulphur dioxide (Negative control), CIPRO(+VE)=Ciprofloxacin (Positive control),CHLO(+VE)= Chloramphenicol (Positive control), Values are means of duplicate readings. Means followed by different superscript letters in the table above are significantly different at P<0.0001.

4.3.3 Comparative analysis of *in vitro* results of disc diffusion and microdilution method

Anti-salmonella activity of plant extracts by disc diffusion and microdilution methods were compared. The nine active plant extracts showed different value of anti-salmonella activity against test isolates. Ethyl acetate extract of *W. ugandensis* stem bark gave the lowest MIC value of 0.031mg/ml against *S. ser. Typhi* (ATCC 13347), *S. ser. Typhi* (ATCC 43579), and *S. ser. Typhimurium* (ATCC 1408) with zones of inhibition of 6, 7 and 7.33mm respectively. The extract showed MIC of 0.061 mg/ml against *S. enterica* (ATCC 2162) with inhibition zone of 6 mm. The *T. diversifolia* extracts had anti-salmonella activity against all the tested clinical isolates. Methanol extract of the leaf showed activity with MIC values of 0.031, 0.24, 0.448 and 0.98mg/ml against *S. ser. Typhi* (ATCC 43579), *S. ser. Typhi* (ATCC 13347), *S. ser. Typhimurium* (ATCC 1408) and *S.enterica* (ATCC 2162) with inhibition zones of 11.5, 11, 11.67 and 7.33 respectively.

4.4 Acute and sub-chronic toxicity studies of mice treated with plant extracts

4.4.1 Acute toxicity studies

In acute toxicity study, TDLE, TDFH, TDLM, TDLH, TDFE, WURE, WURH, WUSBE, WUSBH, up to 2000 mg/kg body weight administered orally exhibited no toxic effect or death in mice. No mortality was evident in mice. The animals did not show any changes in general behavior or other physiological activities. After oral administration of the plant extracts to test animals, it was observed that, between 10-15 minutes, all the mice were active, fur was smooth and feeding was normal. In addition, the animals were breathing normally without any difficulty and with no sign of toxicity effect. It was observed that animals treated with 2000mg/kg (single dose) of plant extracts on average increased weight as compared to control animals. There was no significant difference among the plant extracts administered to animals under experiment ($p<0.0001$) and all survived for the entire 14 days of observation.

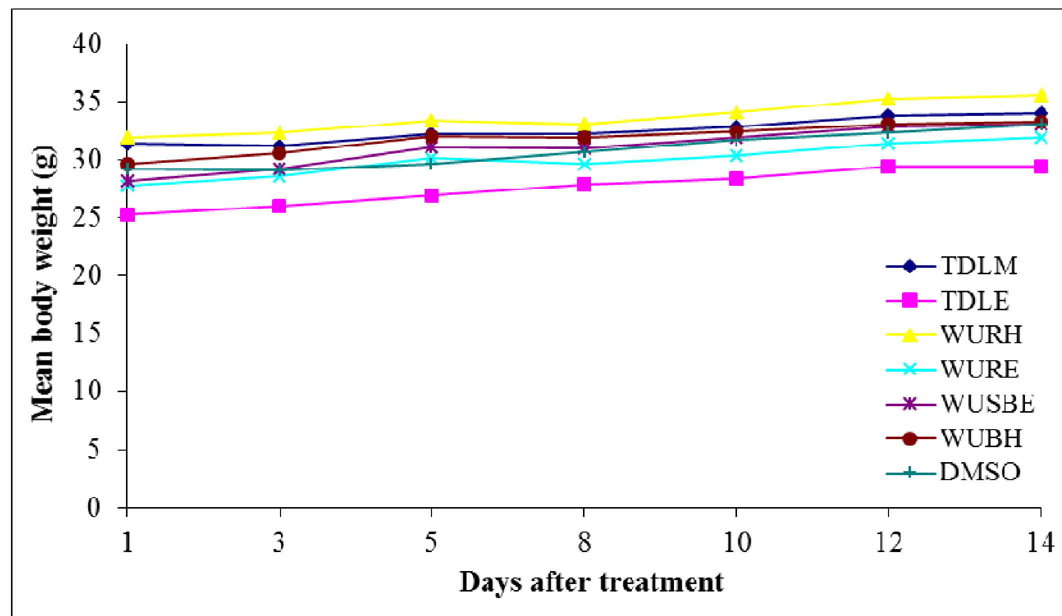


Figure 4.1: Mean body weights (g) of mice treated with plant extracts at 2000 mg/kg

Packed Cell Volume (PCV) analysis gave results that were consistent with observations made on body weights of treated animals. Figure 4.2 shows PCV of animals treated with 2000 mg/kg of plant extracts and on average PCV was in range of 42.2-50.6% which was fairly within the reference values of 42-52% for males. The animals treated with DMSO had PCV in the range of 42.2-44.8%.

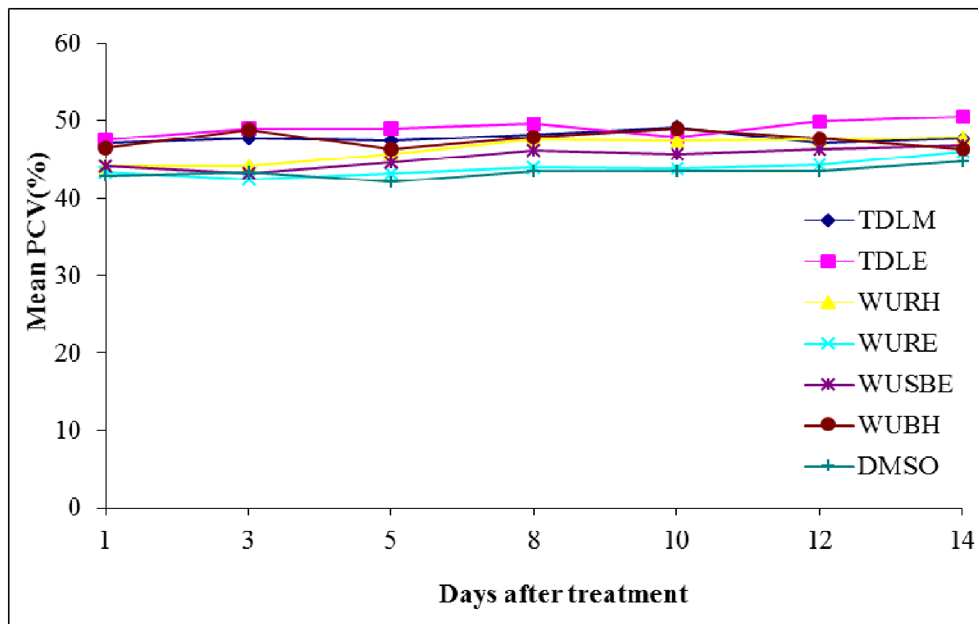
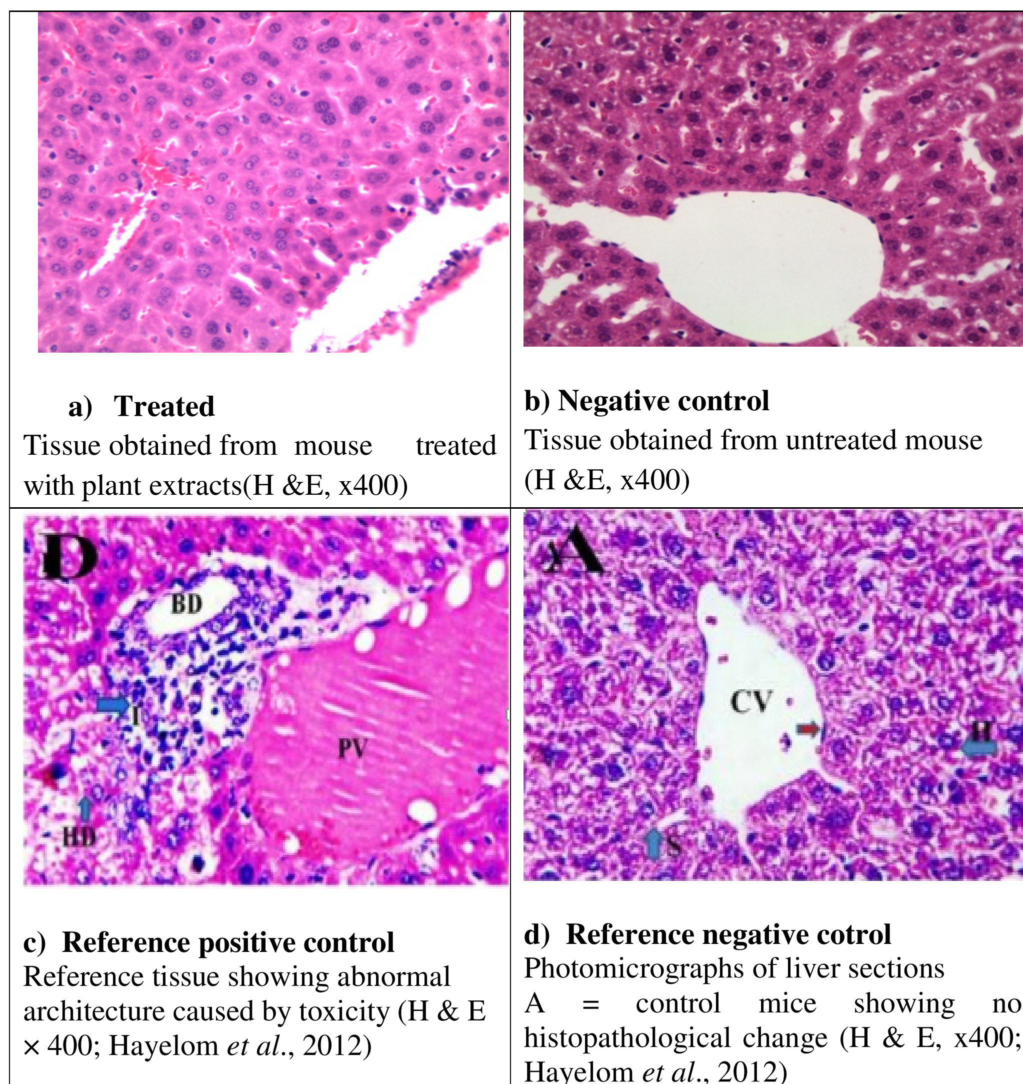


Figure 4.2: Mean PCV of mice treated plant extracts at 2000mg/kg

After 14 days of experiment, the surviving animals were sacrificed in Carbon IV oxide (CO_2) chamber and their liver and kidney were excised. Histopathology revealed no abnormalities after treating with methanol, ethyl acetate and hexane extracts.

Plates 4.1-4.10 show longitudinal sections of the liver and kidney from mice administered with methanol, ethyl acetate and hexane extracts of *T. diversifolia* and *W. ugandensis*. Each plate has (a), (b), (c) and (d) to represent treatments, negative control (untreated), reference positive control and reference negative control respectively. Plates; 4.1a, 4.3a, 4.5a, 4.7a and 4.9a are treatments and show no abnormalities as like in plates; 4.1b, 4.3b, 4.5b, 4.7b and 4.9b for the untreated controls. Plates; 4.1c, 4.3c, 4.5c, 4.7c and 4.9c are the reference positive controls and show inflammations around bile duct and portal vein and vacuolar degenerations of the liver (400mg/kg body weight/day of methanol root extracts of *Clerodendrum myricoides*). Plates; 4.1d, 4.3d, 4.5d, 4.7d and 4.9d are the reference negative controls and show no histological change of liver (Hayelom *et al*., 2012).

Plates; 4.2c, 4.4c, 4.6c, 4.8c and 4.10c are reference positive control for the kidney and show haemorrhage and signs in congestion of the glomeruli (1630mg/kg body weight/day of methanol root extract of *Clerodendrum myricoides*). In addition, Plates; 4.2d, 4.4d, 4.6d, 4.8d and 4.10d are reference negative control for the kidney and show no histopathological changes (Hayelom *et al*., 2012). Histopathology showed no detectable abnormalities of the tissues from treated mice when compared to controls at x400 magnification.



a) Treated
Tissue obtained from mouse treated with plant extracts(H &E, x400)

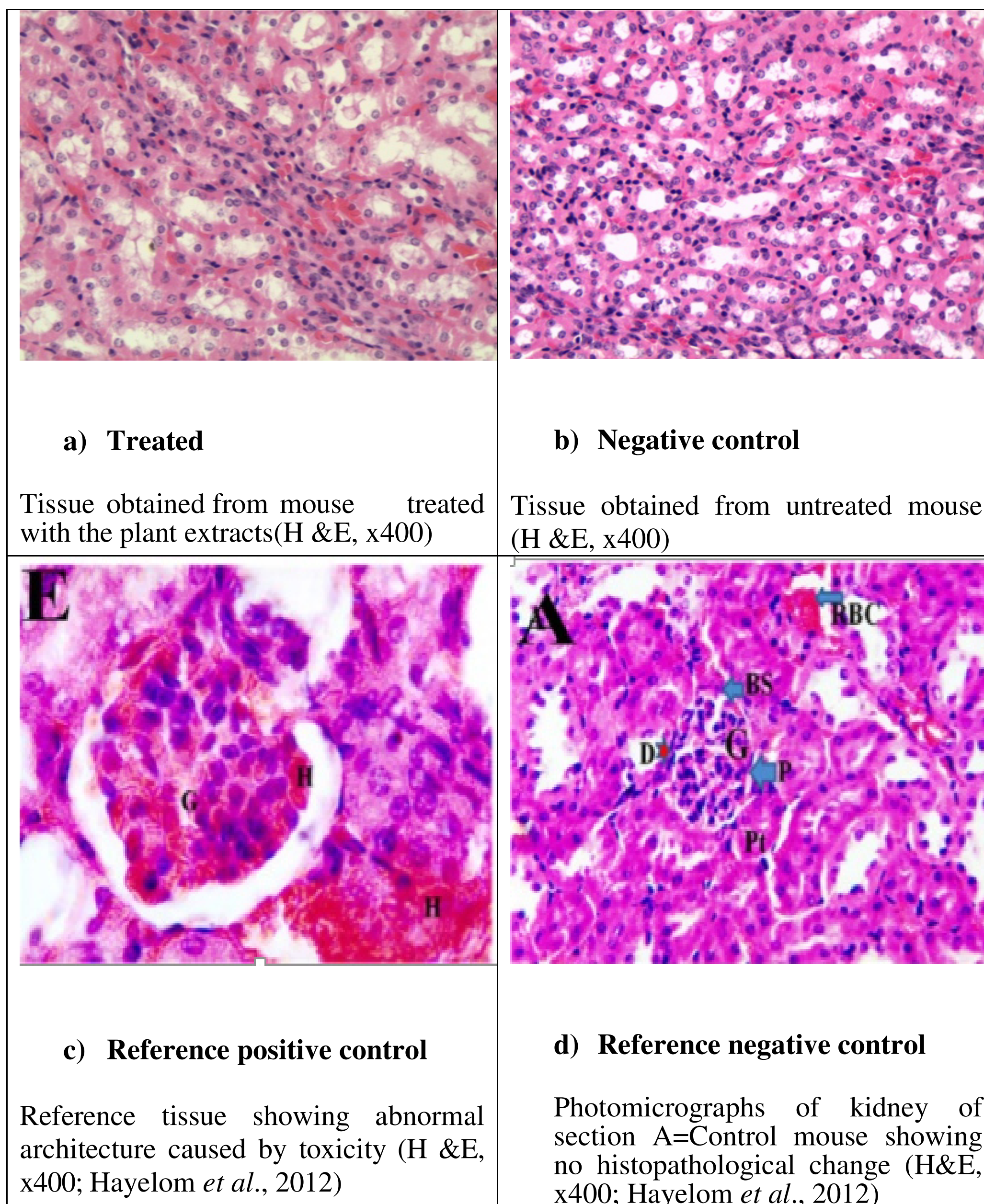
b) Negative control
Tissue obtained from untreated mouse (H &E, x400)

c) Reference positive control
Reference tissue showing abnormal architecture caused by toxicity (H & E × 400; Hayelom *et al*., 2012)

d) Reference negative cotrol
Photomicrographs of liver sections
A = control mice showing no histopathological change (H & E, x400; Hayelom *et al*., 2012)

Plate 4.1: Longitudinal section of liver of mouse treated with methanol extracts of *T. diversifolia* leaf at 2000mg/kg.

(a) and (b) shows the histological status of the liver tissues of both the treated and control mice where normal cellular architecture with prominent central vain was shown which indicates, that the extract did not cause damage to livers when used for therapeutic purpose.(c) Positive control indicating inflammations around bile duct and portal vein (I) and vacuolar degenerations (HD) in mice treated at 400mg/kg body weight/day of methanol root extracts of *Clerodendrum myricoides*((d) Normal control, photomicrograph of liver section showing no histological change. Red arrow in positive control indicates endothelial cells. BD = bile duct, CV=Central vein, HD=Hydropic degeneration, I=inflammatory cells, PV=Portal vein.



a) Treated

Tissue obtained from mouse treated with the plant extracts(H &E, x400)

b) Negative control

Tissue obtained from untreated mouse (H &E, x400)

c) Reference positive control

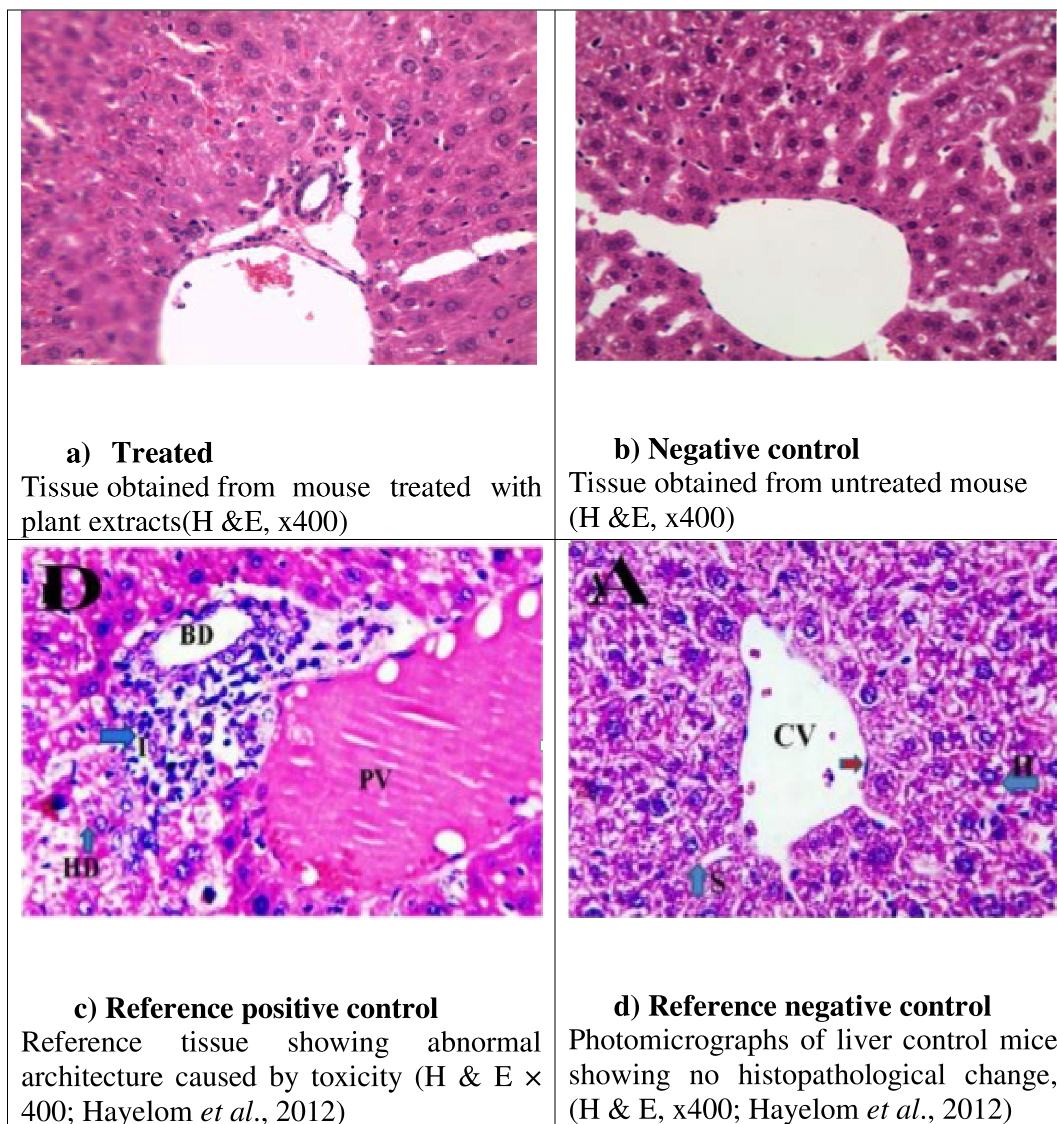
Reference tissue showing abnormal architecture caused by toxicity (H &E, x400; Hayelom *et al*., 2012)

d) Reference negative control

Photomicrographs of kidney of section A=Control mouse showing no histopathological change (H&E, x400; Hayelom *et al*., 2012)

Plate 4.2: Longitudinal section of kidney of mouse treated with methanol extracts of *T. diversifolia* leaf at 2000 mg/kg.

(a) and (b) are light microscopic sections of the kidney showing no histological changes visible in sections of treated and untreated mice. (c) Positive control Indicating haemorrhage and signs in congestion of the glomeruli of the kidney in mice treated with 1630mg/kg body weight/day of methanol root extract of *Clerodendrum myricoides*. (d) Normal control photomicrographs of kidney indicating there are no histopathological changes visible in the sections of the control mice and red arrow indicates macula densa. D== Distal convoluted tubules, G= Glomeruli, H= Haemorrhage, P= Podocytes, Pt= Proximal convoluted tubules, RBC = Red blood cell.



a) Treated
Tissue obtained from mouse treated with plant extracts(H &E, x400)

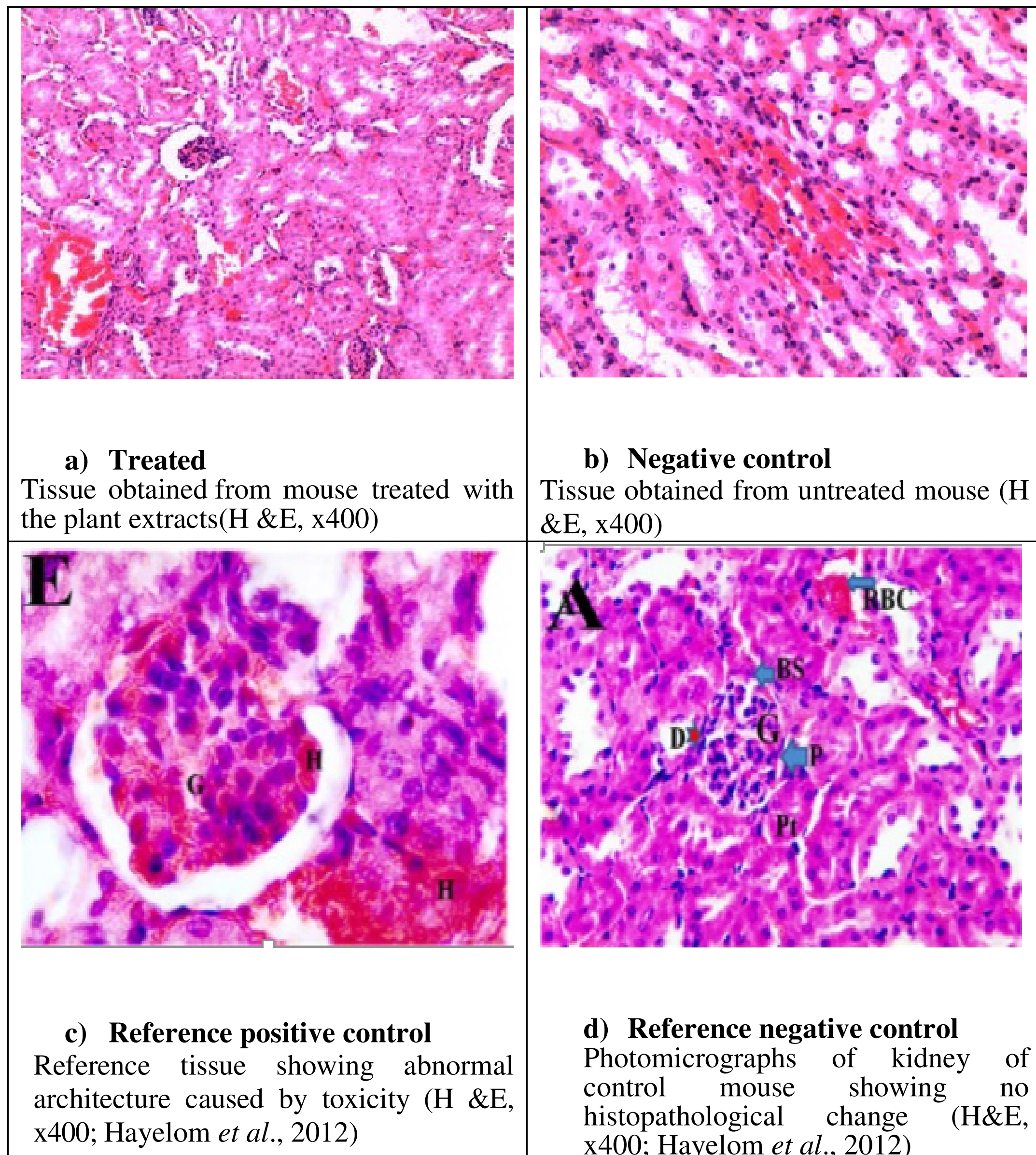
b) Negative control
Tissue obtained from untreated mouse (H &E, x400)

c) Reference positive control
Reference tissue showing abnormal architecture caused by toxicity (H & E × 400; Hayelom *et al*., 2012)

d) Reference negative control
Photomicrographs of liver control mice showing no histopathological change, (H & E, x400; Hayelom *et al*., 2012)

Plate 4.3: Longitudinal section of the liver of mouse treated with ethyl acetate extracts of *T. diversifolia* leaf at 2000 mg/kg.

(a) and (b) shows the histological status of the liver tissues of both the treated and control mice where normal cellular architecture with prominent central vain was shown which indicates, that the extract did not cause damage to livers when used for therapeutic purpose.(c) Positive control indicating inflammations around bile duct and portal vein (I) and vacuolar degenerations (HD) in mice treated at 400mg/kg body weight/day of methanol root extracts of *Clerodendrum myricoides*((d) Normal control, photomicrograph of liver section showing no histological change. Red arrow in positive control indicates endothelial cells. BD = bile duct, CV=Central vein, HD=Hydropic degeneration, I=inflammatory cells, PV=Portal vein.



a) Treated
Tissue obtained from mouse treated with the plant extracts(H &E, x400)

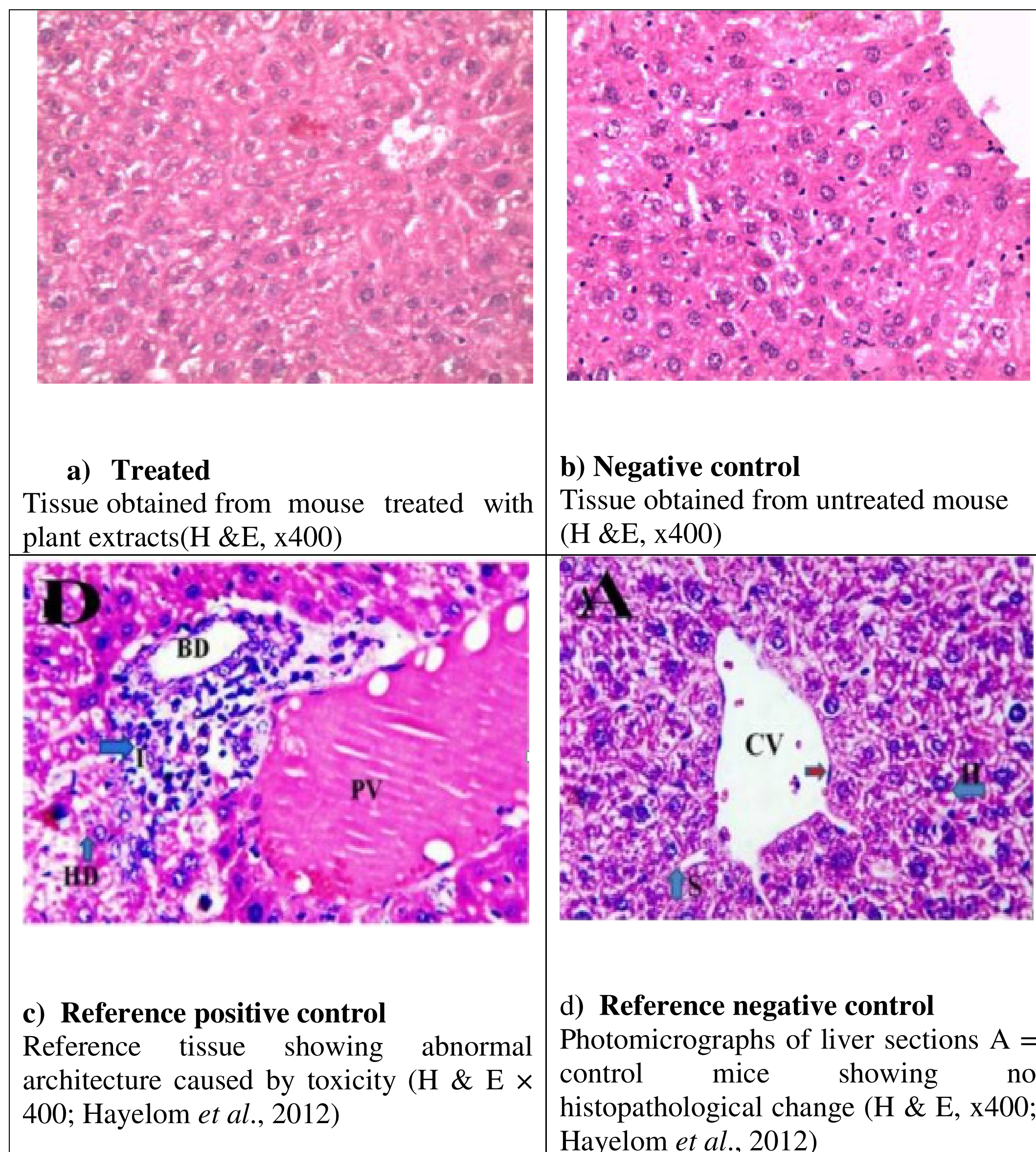
b) Negative control
Tissue obtained from untreated mouse (H &E, x400)

c) Reference positive control
Reference tissue showing abnormal architecture caused by toxicity (H &E, x400; Hayelom *et al*., 2012)

d) Reference negative control
Photomicrographs of kidney of control mouse showing no histopathological change (H&E, x400; Hayelom *et al*., 2012)

Plate 4.4: Longitudinal section of kidney of mouse treated with ethyl acetate extracts of *T. diversifolia* leaf at 2000 mg/kg.

(a) and (b) are light microscopic sections of the kidney showing no histological changes visible in sections of treated and untreated mice. (c) Positive control Indicating haemorrhage and signs in congestion of the glomeruli of the kidney in mice treated with 1630mg/kg body weight/day of methanol root extract of *Clerodendrum myricoides*. (d) Normal control photomicrographs of kidney indicating there are no histopathological changes visible in the sections of the control mice and red arrow indicates macula densa. D= Distal convoluted tubules, G= Glomeruli, H= Haemorrhage, P= Podocytes, Pt= Proximal convoluted tubules, RBC = Red blood cell.



a) Treated
Tissue obtained from mouse treated with plant extracts(H &E, x400)

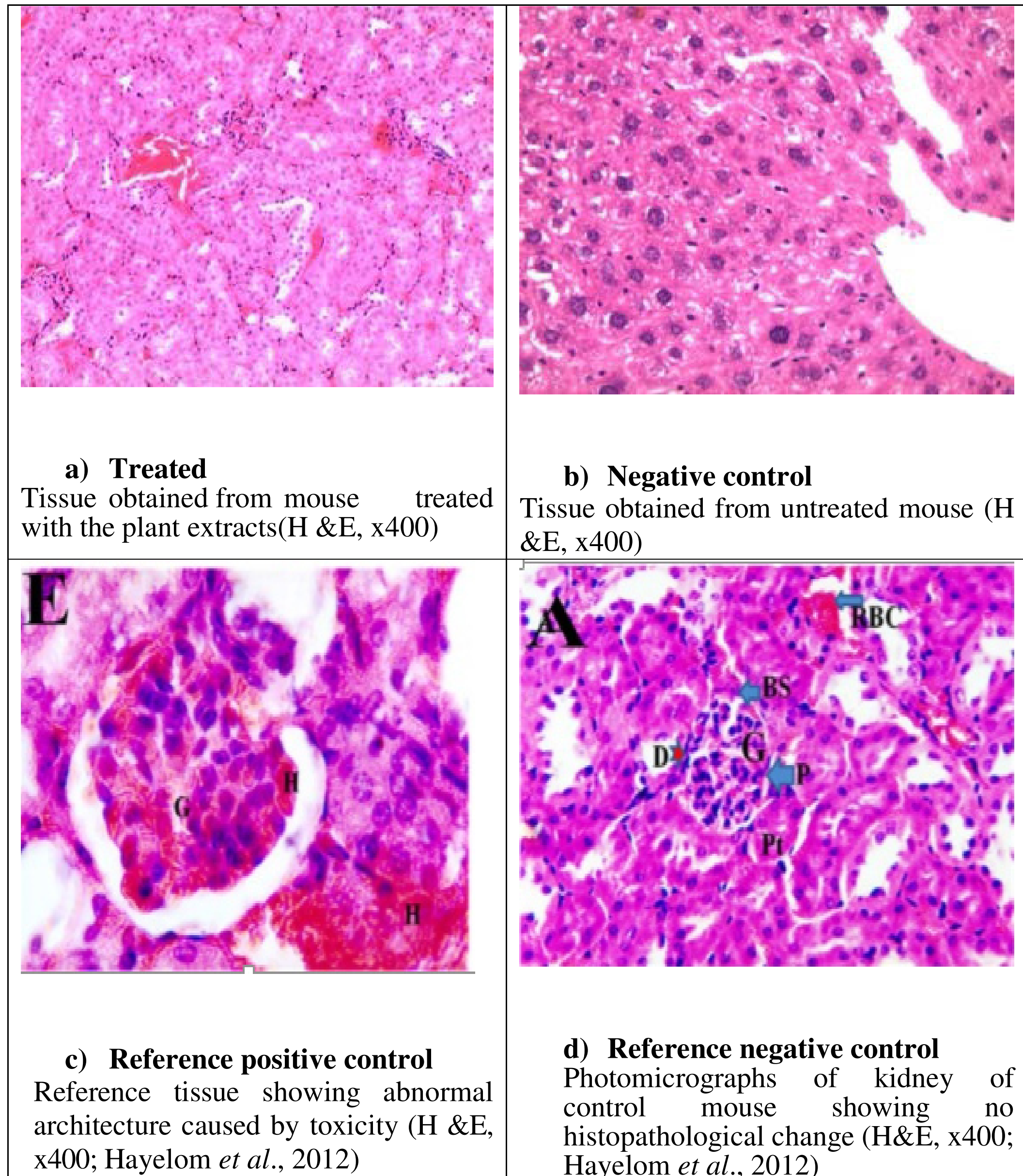
b) Negative control
Tissue obtained from untreated mouse (H &E, x400)

c) **Reference positive control**
Reference tissue showing abnormal architecture caused by toxicity (H & E × 400; Hayelom *et al*., 2012)

d) **Reference negative control**
Photomicrographs of liver sections A = control mice showing no histopathological change (H & E, x400; Hayelom *et al*., 2012)

Plate 4.5: Longitudinal section of liver of mouse treated with hexane extracts of *T. diversifolia* flower at 2000 mg/kg.

(a) and (b) shows the histological status of the liver tissues of both the treated and control mice where normal cellular architecture with prominent central vain was shown which indicates, that the extract did not cause damage to livers when used for therapeutic purpose.(c) Positive control indicating inflammations around bile duct and portal vein (I) and vacuolar degenerations (HD) in mice treated at 400mg/kg body weight/day of methanol root extracts of *Clerodendrum myricoides*((d) Normal control, photomicrograph of liver section showing no histological change. Red arrow in positive control indicates endothelial cells. BD = bile duct, CV=Central vein, HD=Hydropic degeneration, I=inflammatory cells, PV=Portal vein.



a) Treated
Tissue obtained from mouse treated with the plant extracts(H &E, x400)

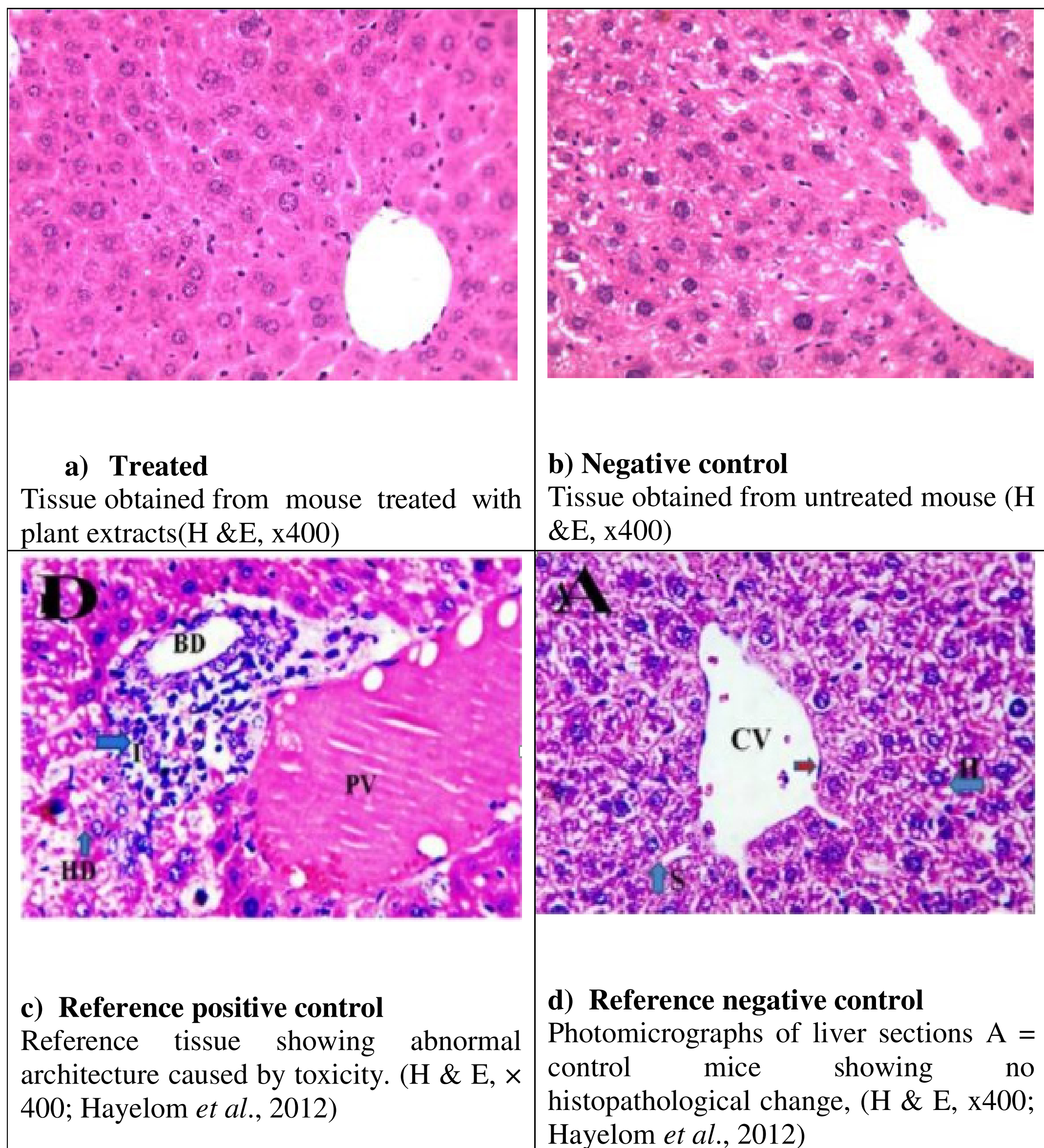
b) Negative control
Tissue obtained from untreated mouse (H &E, x400)

c) Reference positive control
Reference tissue showing abnormal architecture caused by toxicity (H &E, x400; Hayelom *et al*., 2012)

d) Reference negative control
Photomicrographs of kidney of control mouse showing no histopathological change (H&E, x400; Hayelom *et al*., 2012)

Plate 4.6: Longitudinal section of Kidney of mouse treated with hexane extracts of *T. diversifolia* flower at 2000 mg/kg.

(a) and (b) are light microscopic sections of the kidney showing no histological changes visible in sections of treated and untreated mice. (c) Positive control Indicating haemorrhage and signs in congestion of the glomeruli of the kidney in mice treated with 1630mg/kg body weight/day of methanol root extract of *Clerodendrum myricoides*. (d) Normal control photomicrographs of kidney indicating there are no histopathological changes visible in the sections of the control mice and red arrow indicates macula densa. D= Distal convoluted tubules, G= Glomeruli, H= Haemorrhage, P= Podocytes, Pt= Proximal convoluted tubules, RBC = Red blood cell.



a) Treated
Tissue obtained from mouse treated with plant extracts(H &E, x400)

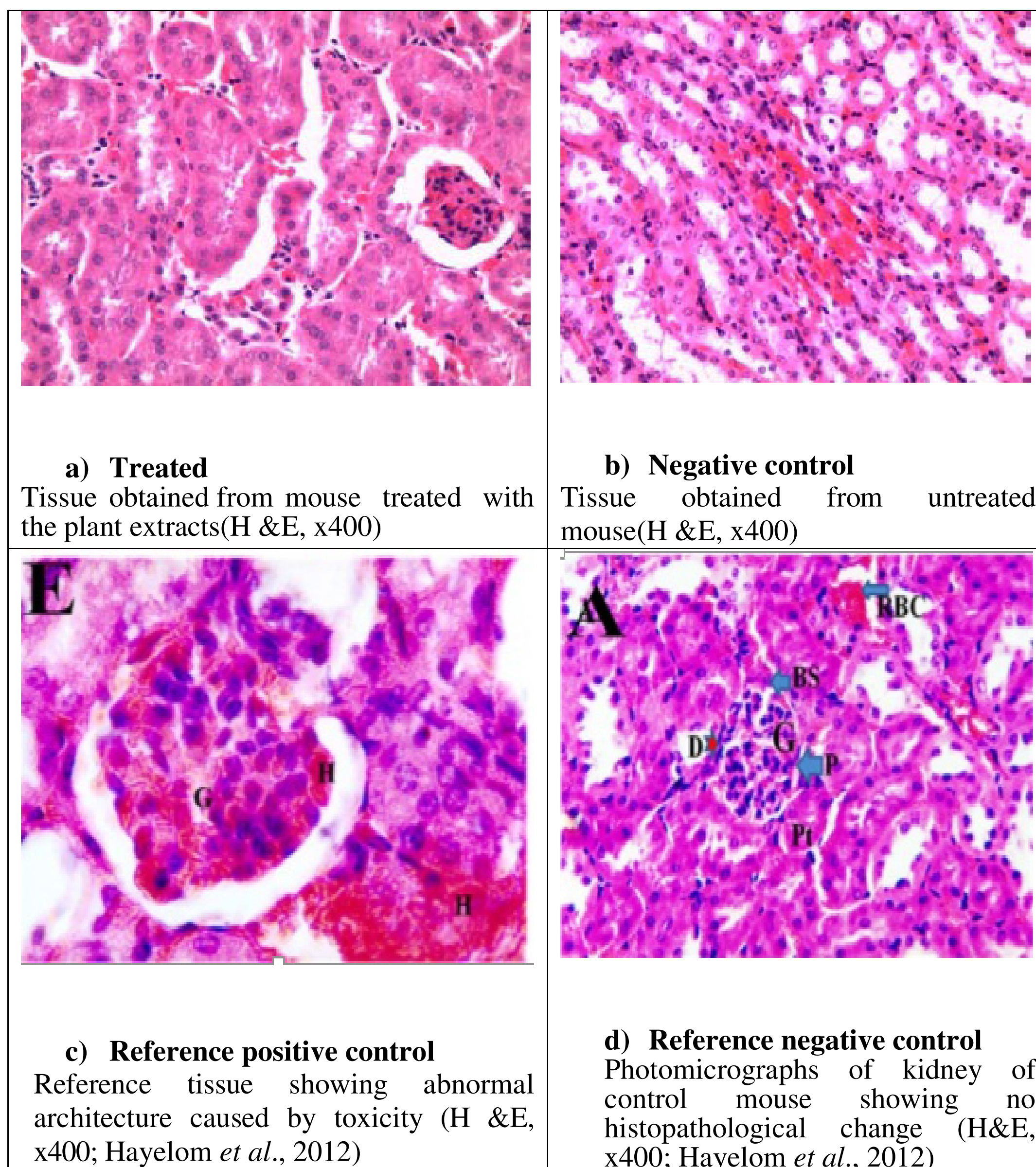
b) Negative control
Tissue obtained from untreated mouse (H &E, x400)

c) Reference positive control
Reference tissue showing abnormal architecture caused by toxicity. (H & E, × 400; Hayelom *et al*., 2012)

d) Reference negative control
Photomicrographs of liver sections A = control mice showing no histopathological change, (H & E, x400; Hayelom *et al*., 2012)

Plate 4.7: Longitudinal section of liver of mouse treated with ethyl acetate extracts of *W. ugandensis* bark at 2000 mg/kg.

(a) and (b) shows the histological status of the liver tissues of both the treated and control mice where normal cellular architecture with prominent central vain was shown which indicates, that the extract did not cause damage to livers when used for therapeutic purpose.(c) Positive control indicating inflammations around bile duct and portal vein (I) and vacuolar degenerations (HD) in mice treated at 400mg/kg body weight/day of methanol root extracts of *Clerodendrum myricoides* (d) Normal control, photomicrograph of liver section showing no histological change. Red arrow in positive control indicates endothelial cells. BD = bile duct, CV=Central vein, HD=Hydropic degeneration, I=inflammatory cells, PV=Portal vein.



a) Treated
Tissue obtained from mouse treated with the plant extracts(H &E, x400)

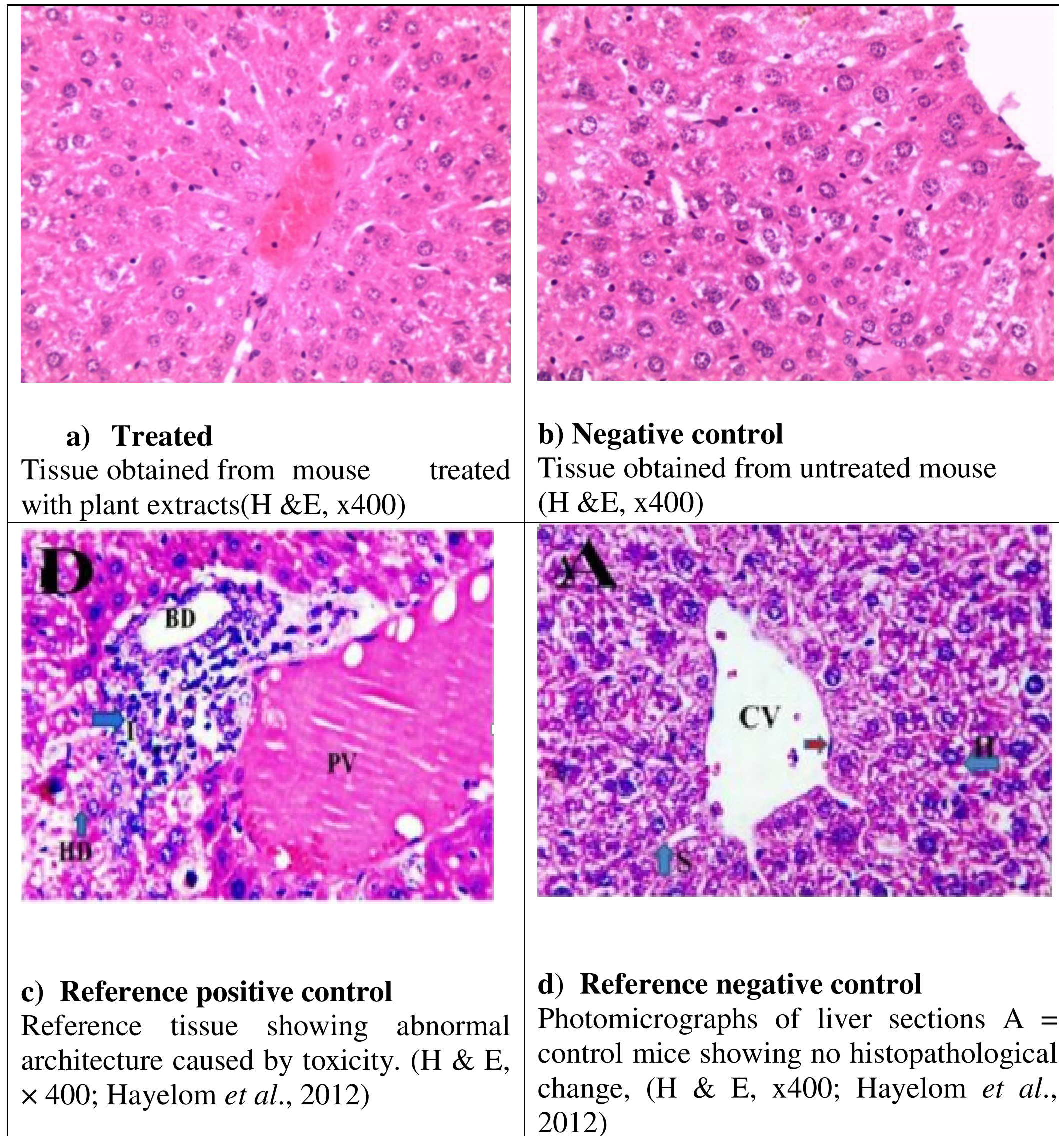
b) Negative control
Tissue obtained from untreated mouse(H &E, x400)

c) Reference positive control
Reference tissue showing abnormal architecture caused by toxicity (H &E, x400; Hayelom *et al*., 2012)

d) Reference negative control
Photomicrographs of kidney of control mouse showing no histopathological change (H&E, x400; Hayelom *et al*., 2012)

Plate 4.8: Longitudinal section of Kidney of mouse treated with ethyl acetate extracts of *W. ugandensis* bark at 2000 mg/kg.

(a) and (b) are light microscopic sections of the kidney showing no histological changes visible in sections of treated and untreated mice. (c) Positive control Indicating haemorrhage and signs in congestion of the glomeruli of the kidney in mice treated with 1630mg/kg body weight/day of methanol root extract of *Clerodendrum myricoides*. (d) Normal control photomicrographs of kidney indicating there are no histopathological changes visible in the sections of the control mice and red arrow indicates macula densa. D= Distal convoluted tubules, G= Glomeruli, H= Haemorrhage, P= Podocytes, Pt= Proximal convoluted tubules, RBC = Red blood cell.



a) Treated
Tissue obtained from mouse treated with plant extracts(H &E, x400)

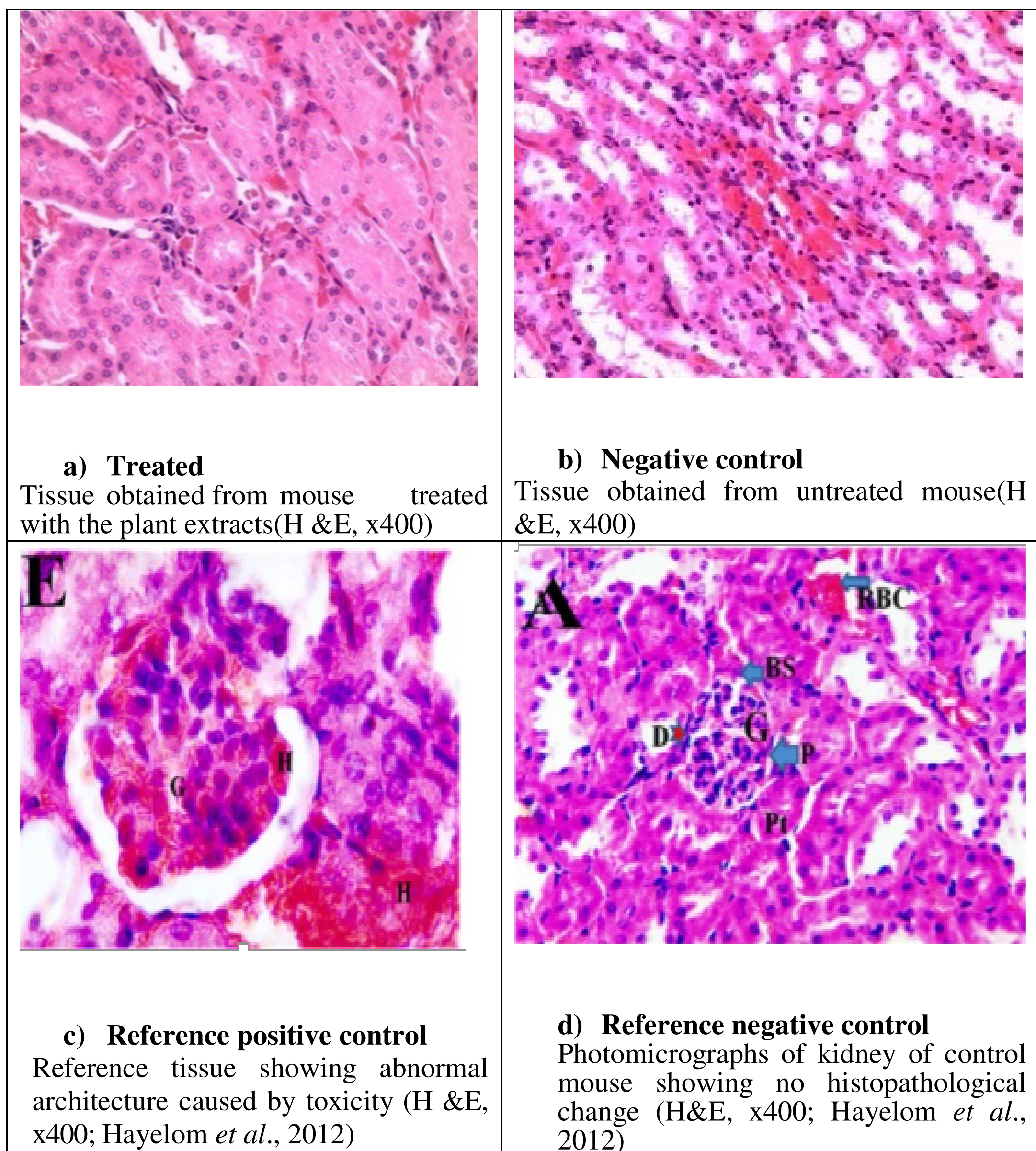
b) Negative control
Tissue obtained from untreated mouse (H &E, x400)

c) Reference positive control
Reference tissue showing abnormal architecture caused by toxicity. (H & E, × 400; Hayelom *et al*., 2012)

d) Reference negative control
Photomicrographs of liver sections A = control mice showing no histopathological change, (H & E, x400; Hayelom *et al*., 2012)

Plate 4.9: Longitudinal section of liver of mouse treated with hexane extracts of *W. ugandensis* root at 2000 mg/kg.

(a) and (b) shows the histological status of the liver tissues of both the treated and control mice where normal cellular architecture with prominent central vain was shown which indicates, that the extract did not cause damage to livers when used for therapeutic purpose.(c) Positive control indicating inflammations around bile duct and portal vein (I) and vacuolar degenerations (HD) in mice treated at 400mg/kg body weight/day of methanol root extracts of *Clerodendrum myricoides* (d) Normal control, photomicrograph of liver section showing no histological change. Red arrow in positive control indicates endothelial cells. BD = bile duct, CV=Central vein, HD=Hydropic degeneration, I=inflammatory cells, PV=Portal vein.



a) Treated
Tissue obtained from mouse treated with the plant extracts(H &E, x400)

b) Negative control
Tissue obtained from untreated mouse(H &E, x400)

c) Reference positive control
Reference tissue showing abnormal architecture caused by toxicity (H &E, x400; Hayelom *et al*., 2012)

d) Reference negative control
Photomicrographs of kidney of control mouse showing no histopathological change (H&E, x400; Hayelom *et al*., 2012)

Plate 4.10: Longitudinal section of kidney of mouse treated with hexane extracts of *W. ugandensis* root at 2000 mg/kg.

(a) and (b) are light microscopic sections of the kidney showing no histological changes visible in sections of treated and untreated mice. (c) Positive control Indicating haemorrhage and signs in congestion of the glomeruli of the kidney in mice treated with 1630mg/kg body weight/day of methanol root extract of *Clerodendrum myricoides*. (d) Normal control photomicrographs of kidney indicating there are no histopathological changes visible in the sections of the control mice and red arrow indicates macula densa. D= Distal convoluted tubules, G= Glomeruli, H= Haemorrhage, P= Podocytes, Pt= Proximal convoluted tubules, RBC = Red blood cell.

4.4.2 Sub-chronic toxicity studies

Mice treated with TDLE, TDFH, TDLM, TDLH, TDFE, WURE, WURH, WUSBE, WUSBH extracts at doses of 300, 600 and 1200mg/kg body weight orally for a period of 30 days, showed normal general behavior, respiratory pattern, skin and fur. Body weight and PCV were also normal (Figure 4.3).

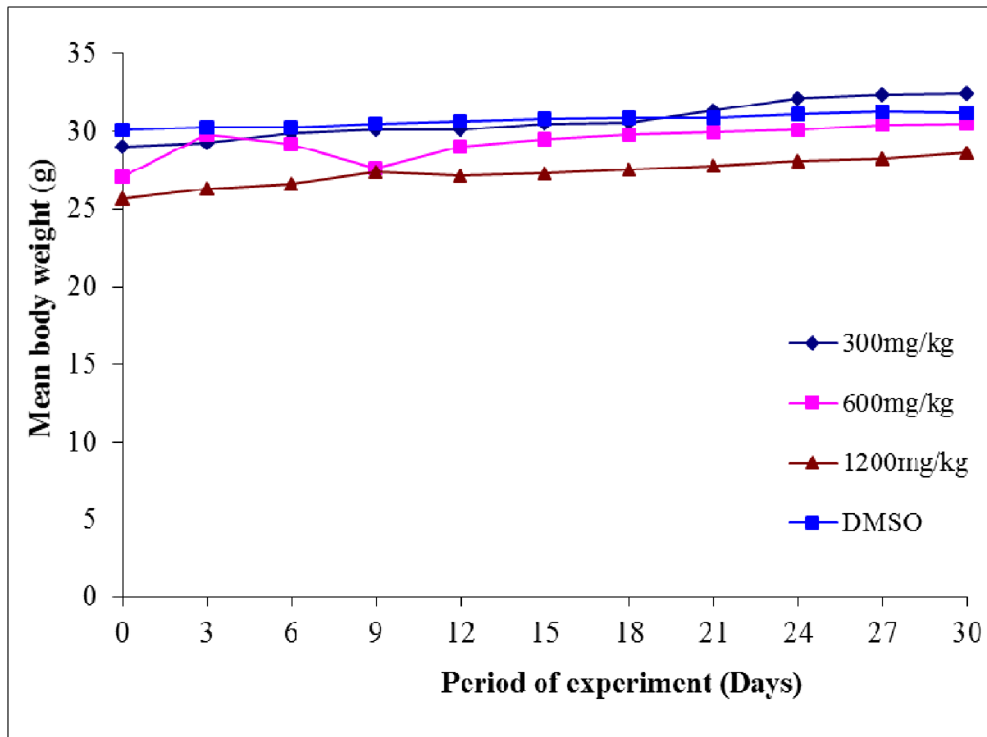


Figure 4.3: Mean body weight (g) of mice treated with TDLM extract

Mean body weight of mice treated with WURH extract was monitored during the period of experiment (Figure 4.4). It was noted that the mice treated with 300, 600 and 1200 mg/kg of hexane extract of *W. ugandensis* root orally, on average demonstrated increase in body weight and survived the entire period of observation. The mice treated with 300mg/kg of extract in first 3 days showed reduced body weight and thereafter gained weight up to the end of experiment. The mice treated with control (DMSO) showed increase of body weight which was comparable with treated mice (Figure 4.4).

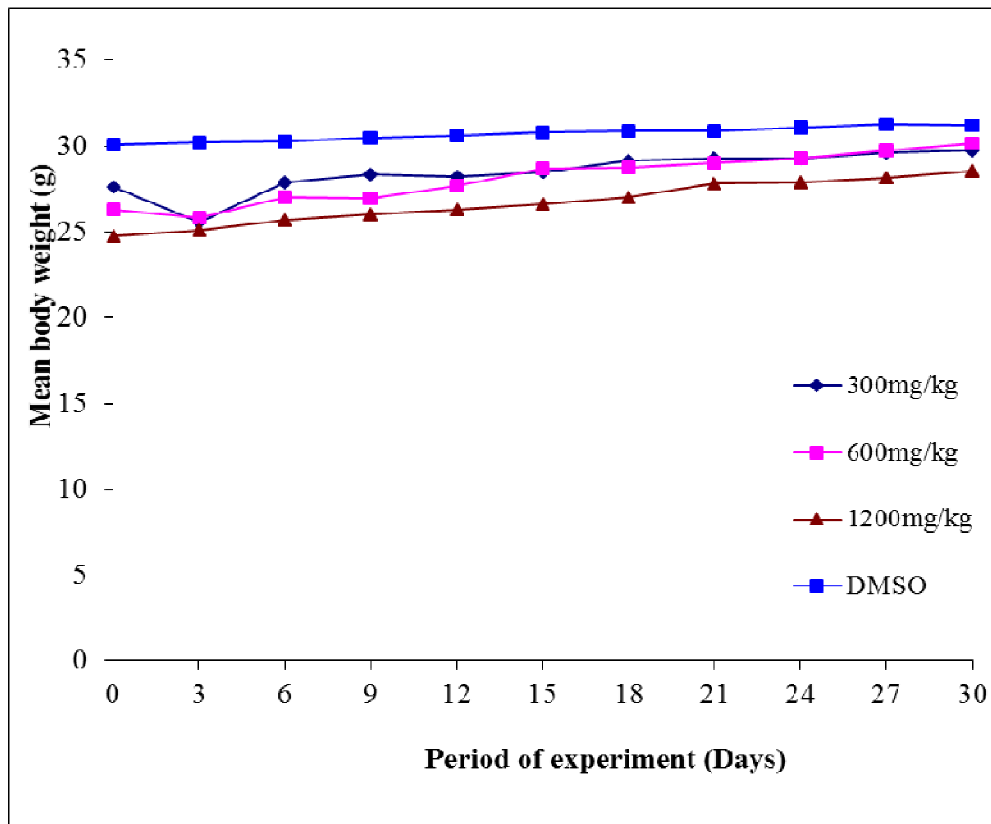


Figure 4.4: Mean body weight (g) of mice treated with WURH extract

Figure 4.5 shows mean body weight of mice orally given WUSBE extract at 3 dose levels (300, 600 and 1200 mg/kg) during the period of the experiment. It was observed that 3 doses of extract given to mice did not affect weight and the mice survived until end of experiment period. There was no significant difference between treated mice and control group that was given DMSO ($p<0.0001$).

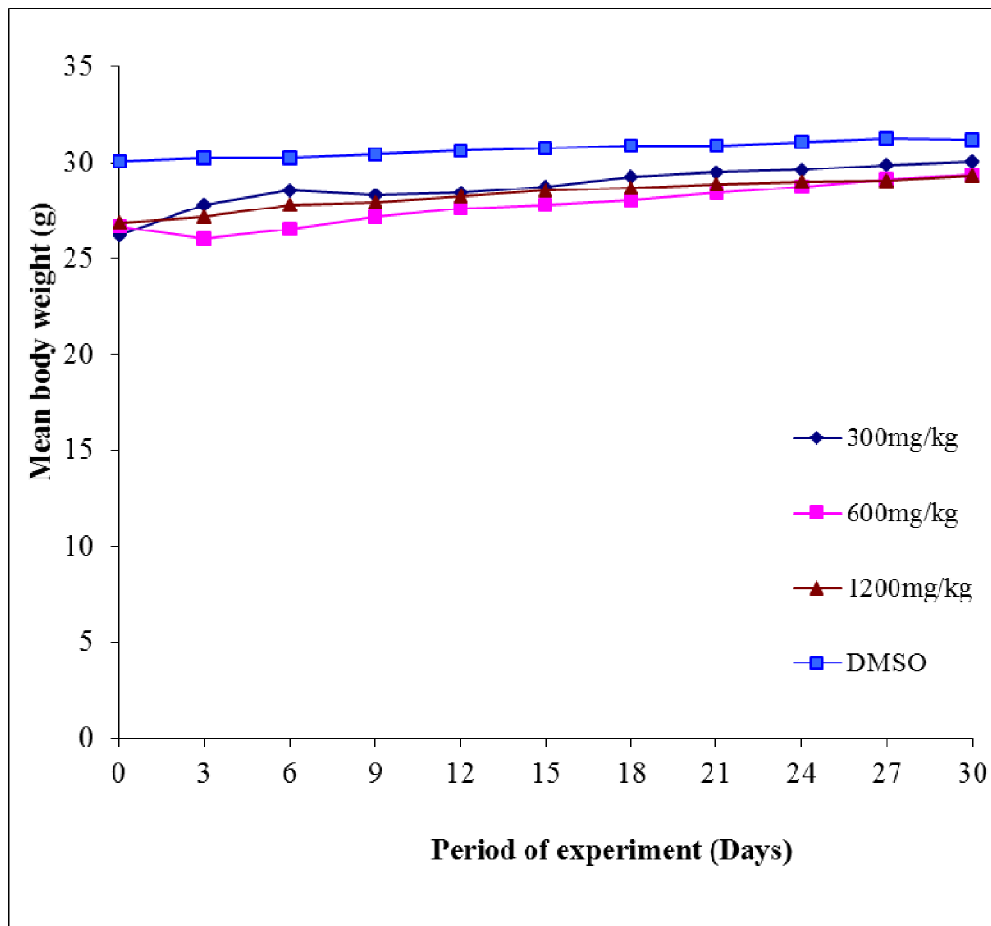


Figure 4.5: Mean body weight (g) of mice treated with WUSBE extract

For sub-chronic toxicity, mean body weight and PCV were monitored as indicators of determining affect by plant extracts. Mice orally treated with TDLM extract at 300, 600 and 1200 mg/kg showed normal levels of PCV during period of experiment (Figure 4.6). PCV analysis gave results that were consistent with the observations made on mean body weight of mice.

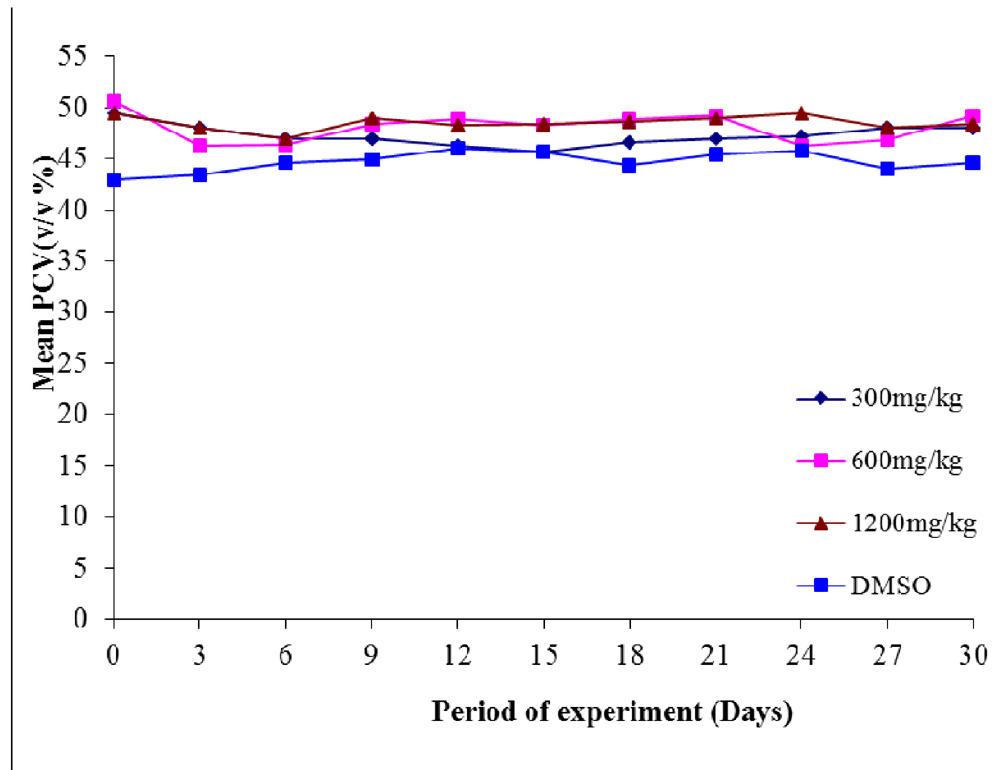


Figure 4.6: Mean PCV of mice treated with TDLM extract

Figure 4.7 shows mean change of PCV levels in mice treated with WURH extract in 3 doses for period of 30 days. The PCV values of all mice administered orally with the plant extract at concentrations of 300, 600 and 1200 mg/kg in the entire period of observation were within the reference limits (42-52%).

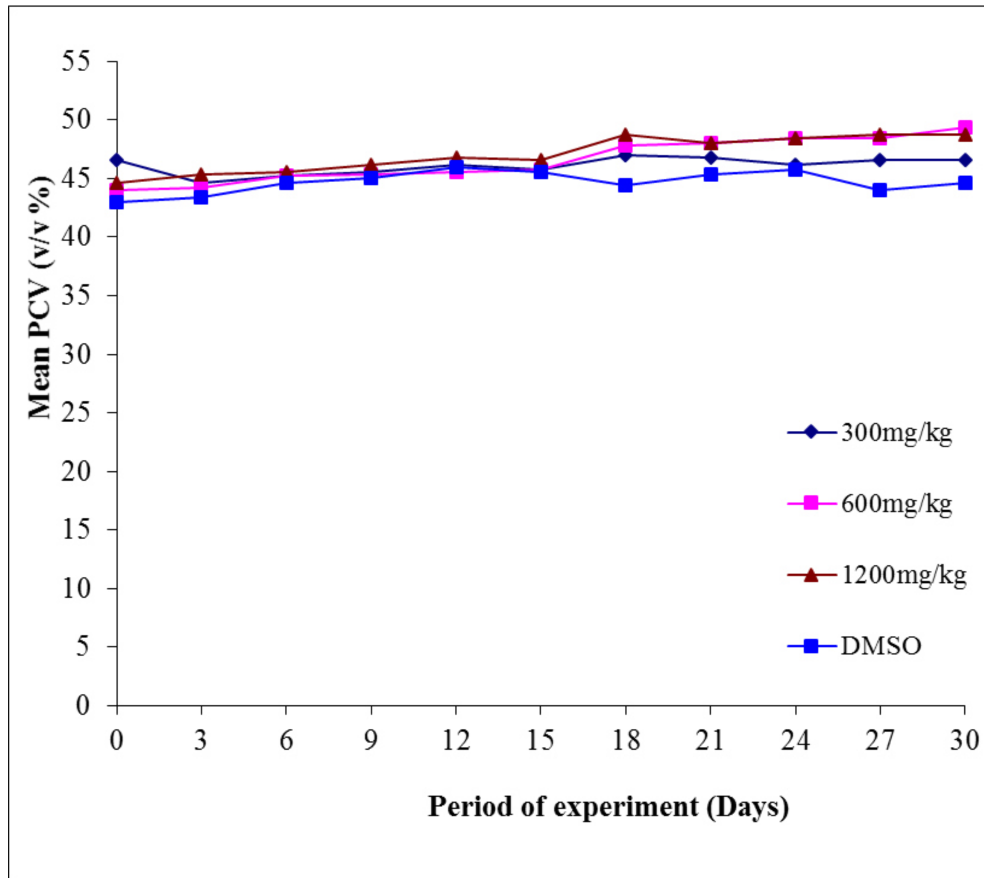


Figure 4.7: Mean PCV of mice treated with WURH extract

Figure 4.8 shows mean PCV levels in mice treated with WUSBE extract for 30 days. The results of PCV indicate that mice were not affected by the plant extract in 3 dose levels and all survived up to the end of observation period. Their PCV values were within reference levels in male mice (42-52%). Similar levels were observed in control group of treated with DMSO (Figure 4-8).

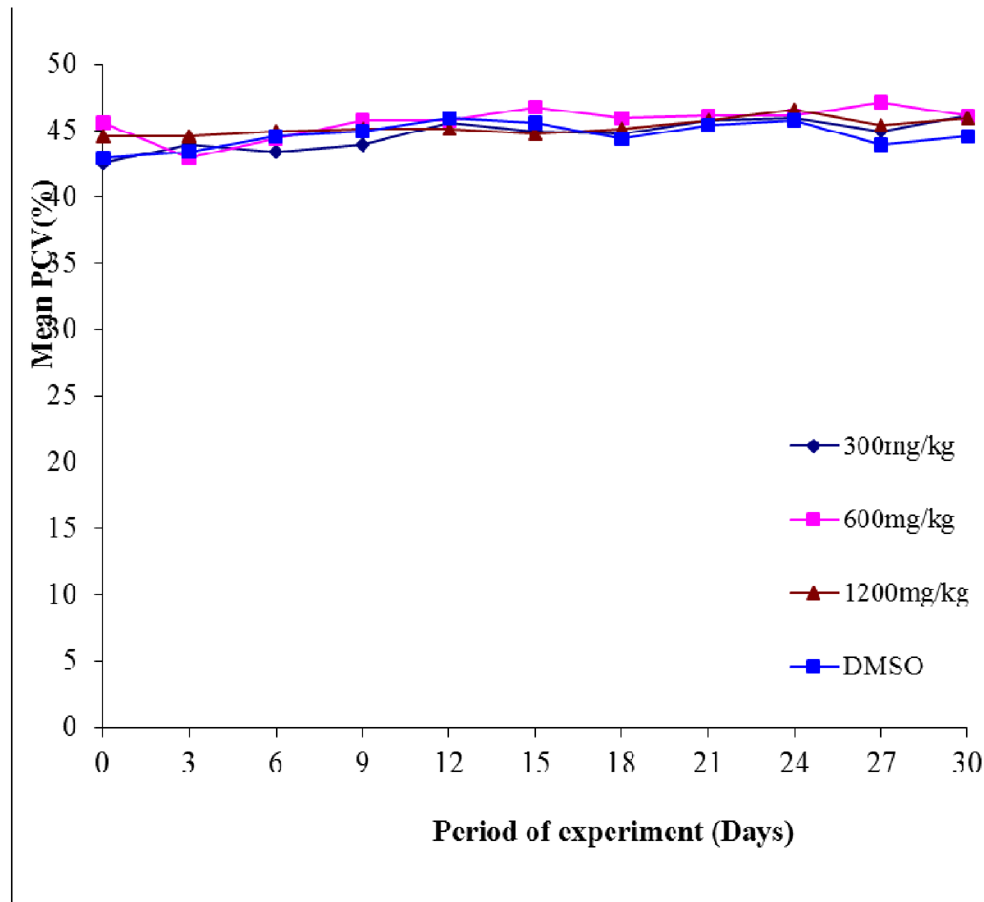


Figure 4.8: Mean PCV of mice treated with WUSBE extract

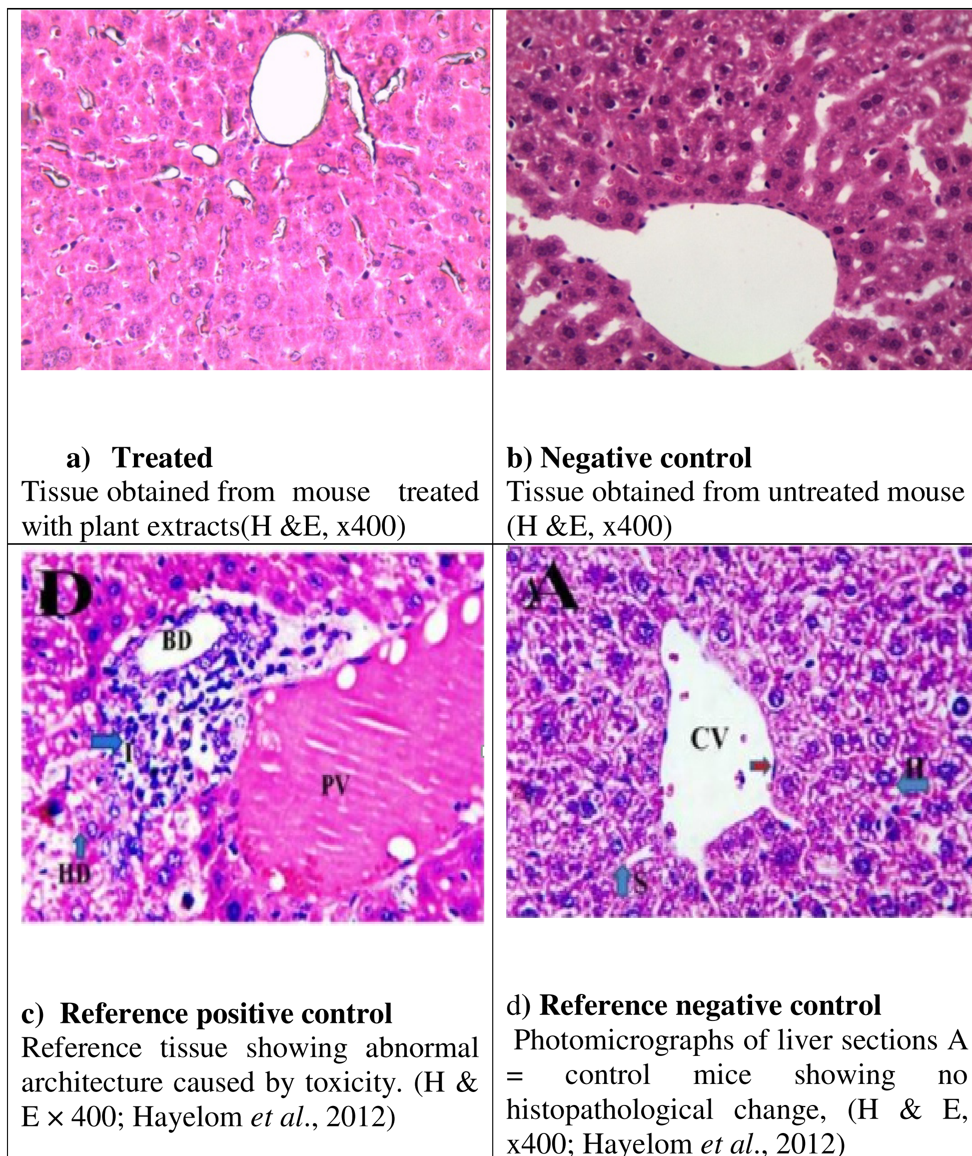
Similarly, mice treated with TDLE, TDFH, TDLH, TDFE, WURE and WUSBH extracts showed same levels of mean body weight and PCV as those treated with TDLM, WURH and WUSBE extracts. No death was recorded in any group for the period of 30 days.

4.4.3 Effect of plant extracts on the liver and kidney in mice

After 30 days of experiment for sub-chronic toxicity study, the surviving mice were sacrificed and their liver and kidney were excised. Histopathology revealed no abnormalities after treating with methanol, ethyl acetate and hexane extracts.

Plates, 4.11-4.20 show histology of liver and kidney tissues treated with 1200 mg/kg body weight extracts and c ontrol group. The histological sections of liver and kidney from control group as well as the treatment showed no histological change. Reference positive control, sections from the liver and kidney of showed inflammations around bile duct and portal vein and vacuolar degenerations (liver) and haemorrhage and signs in congestion of the glomeruli of the kidney.

Similarly, the extracts given at doses of 300mg/kg and 600mg/kg to mice for entire period of experiment survived. Thereafter they were also sacrificed and their liver and kidney tissues excise. Histopathology revealed no abnormalities after treating with methanol, ethyl acetate and hexane extracts.



a) Treated
Tissue obtained from mouse treated with plant extracts(H &E, x400)

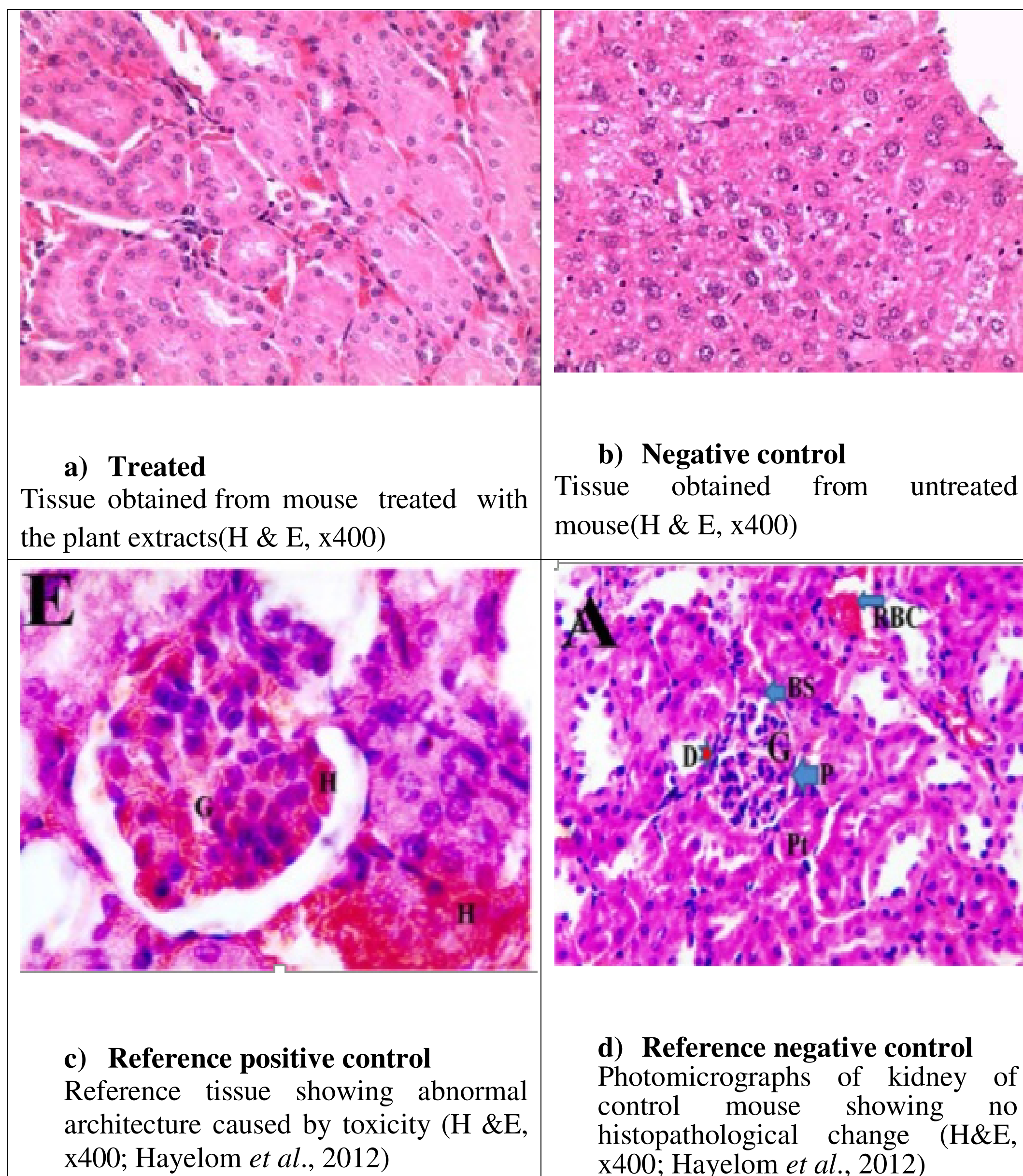
b) Negative control
Tissue obtained from untreated mouse (H &E, x400)

c) Reference positive control
Reference tissue showing abnormal architecture caused by toxicity. (H & E × 400; Hayelom *et al*., 2012)

d) **Reference negative control**
Photomicrographs of liver sections A = control mice showing no histopathological change, (H & E, x400; Hayelom *et al*., 2012)

Plate 4.11: Longitudinal section of liver of mouse treated with methanol extracts of *T. diversifolia* leaf at1200 mg/kg.

(a) and (b) shows the histological status of the liver tissues of both the treated and control mice where normal cellular architecture with prominent central vain was shown which indicates, that the extract did not cause damage to livers when used for therapeutic purpose.(c) Positive control indicating inflammations around bile duct and portal vein (I) and vacuolar degenerations (HD) in mice treated at 400mg/kg body weight/day of methanol root extracts of *Clerodendrum myricoides* (d) Normal control, photomicrograph of liver section showing no histological change. Red arrow in positive control indicates endothelial cells. BD = bile duct, CV=Central vein, HD=Hydropic degeneration, I=inflammatory cells, PV=Portal vein.



a) Treated
Tissue obtained from mouse treated with the plant extracts(H & E, x400)

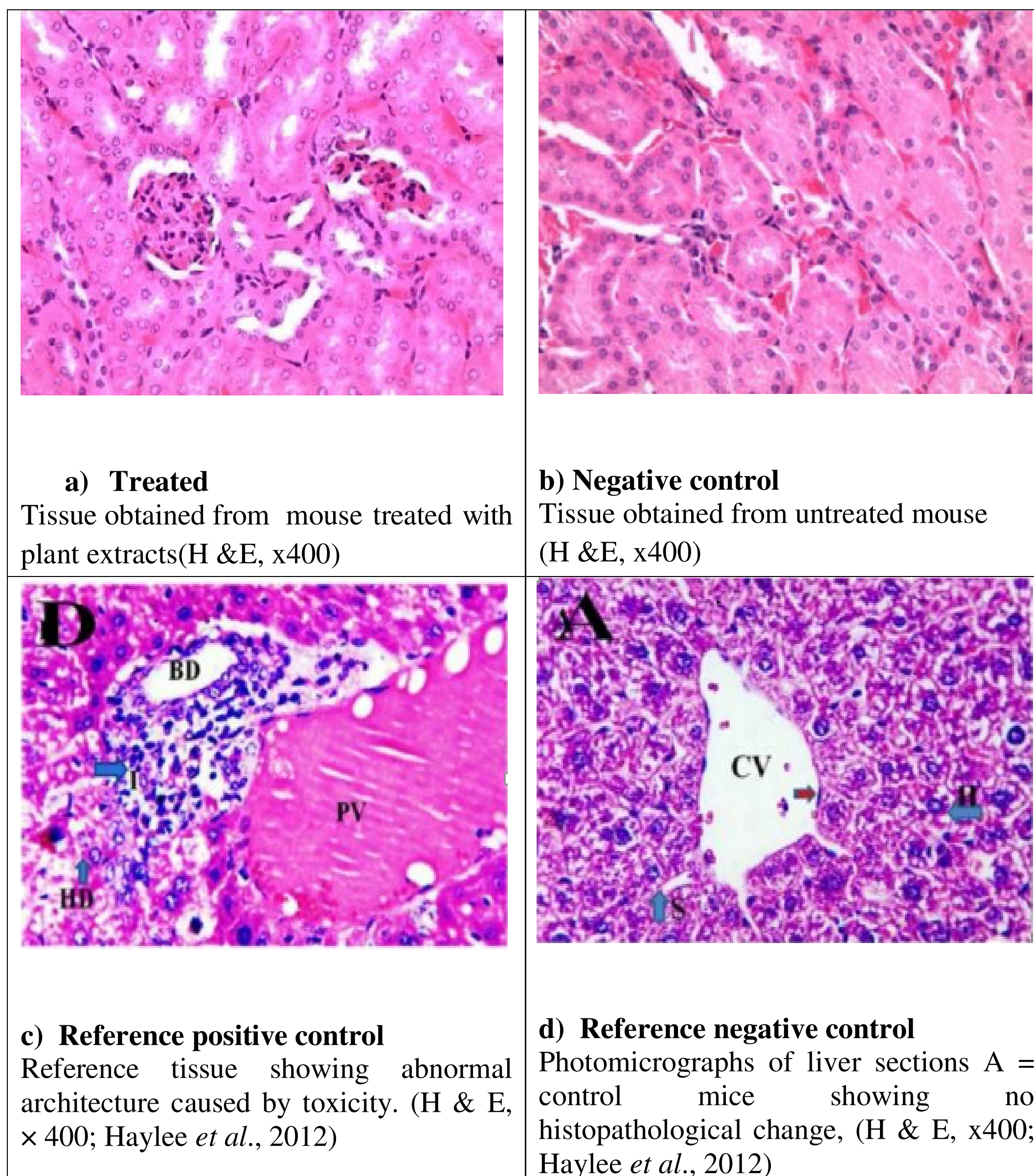
b) Negative control
Tissue obtained from untreated mouse(H & E, x400)

c) Reference positive control
Reference tissue showing abnormal architecture caused by toxicity (H &E, x400; Hayelom *et al*., 2012)

d) Reference negative control
Photomicrographs of kidney of control mouse showing no histopathological change (H&E, x400; Hayelom *et al*., 2012)

Plate 4.12: Longitudinal section of kidney of mouse treated with methanol extracts of *T. diversifolia* leaf at 1200 mg/kg.

(a) and (b) are light microscopic sections of the kidney showing no histological changes visible in sections of treated and untreated mice. (c) Positive control Indicating haemorrhage and signs in congestion of the glomeruli of the kidney in mice treated with 1630mg/kg body weight/day of methanol root extract of *Clerodendrum myricoides*. (d) Normal control photomicrographs of kidney indicating there are no histopathological changes visible in the sections of the control mice and red arrow indicates macula densa. D= Distal convoluted tubules, G= Glomeruli, H= Haemorrhage, P= Podocytes, Pt= Proximal convoluted tubules, RBC = Red blood cell.



a) Treated
Tissue obtained from mouse treated with plant extracts(H &E, x400)

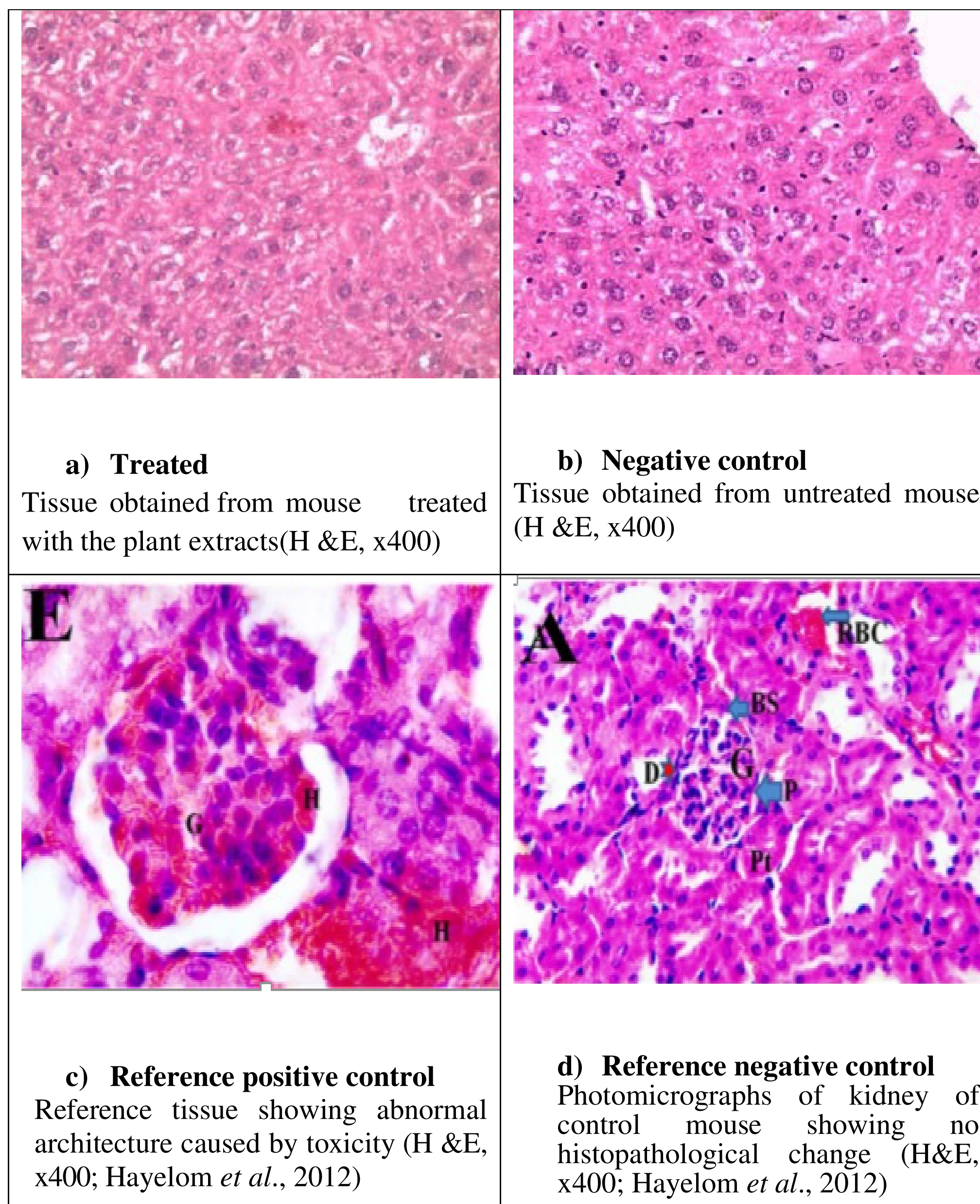
b) Negative control
Tissue obtained from untreated mouse (H &E, x400)

c) Reference positive control
Reference tissue showing abnormal architecture caused by toxicity. (H & E, × 400; Haylee *et al*., 2012)

d) Reference negative control
Photomicrographs of liver sections A = control mice showing no histopathological change, (H & E, x400; Haylee *et al*., 2012)

Plate 4.13: Longitudinal section of liver of mouse treated with ethyl acetate extracts of *T. diversifolia* leaf at 1200 mg/kg.

(a) and (b) shows the histological status of the liver tissues of both the treated and control mice where normal cellular architecture with prominent central vain was shown which indicates, that the extract did not cause damage to livers when used for therapeutic purpose.(c) Positive control indicating inflammations around bile duct and portal vein (I) and vacuolar degenerations (HD) in mice treated at 400mg/kg body weight/day of methanol root extracts of *Clerodendrum myricoides* (d) Normal control, photomicrograph of liver section showing no histological change. Red arrow in positive control indicates endothelial cells. BD = bile duct, CV=Central vein, HD=Hydropic degeneration, I=inflammatory cells, PV=Portal vein.



a) Treated
Tissue obtained from mouse treated with the plant extracts(H &E, x400)

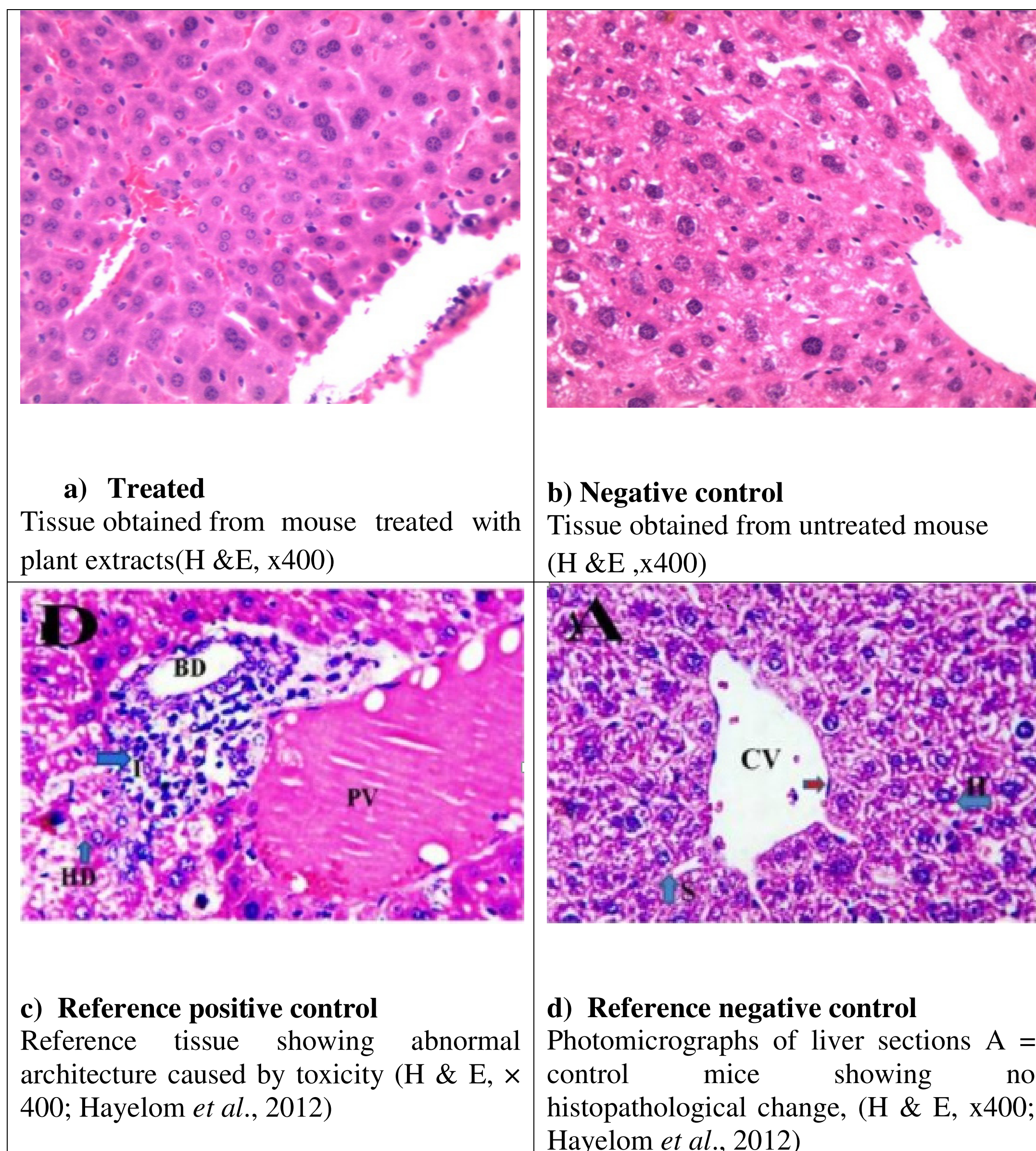
b) Negative control
Tissue obtained from untreated mouse (H &E, x400)

c) Reference positive control
Reference tissue showing abnormal architecture caused by toxicity (H &E, x400; Hayelom *et al*., 2012)

d) Reference negative control
Photomicrographs of kidney of control mouse showing no histopathological change (H&E, x400; Hayelom *et al*., 2012)

Plate 4.14: Longitudinal section of Kidney of mouse treated with ethyl acetate extracts of *T. diversifolia* leaf at 1200 mg/kg.

(a) and (b) are light microscopic sections of the kidney showing no histological changes visible in sections of treated and untreated mice. (c) Positive control Indicating haemorrhage and signs in congestion of the glomeruli of the kidney in mice treated with 1630mg/kg body weight/day of methanol root extract of *Clerodendrum myricoides*. (d) Normal control photomicrographs of kidney indicating there are no histopathological changes visible in the sections of the control mice and red arrow indicates macula densa. D= Distal convoluted tubules, G= Glomeruli, H= Haemorrhage, P= Podocytes, Pt= Proximal convoluted tubules, RBC = Red blood cell.



a) Treated
Tissue obtained from mouse treated with plant extracts(H &E, x400)

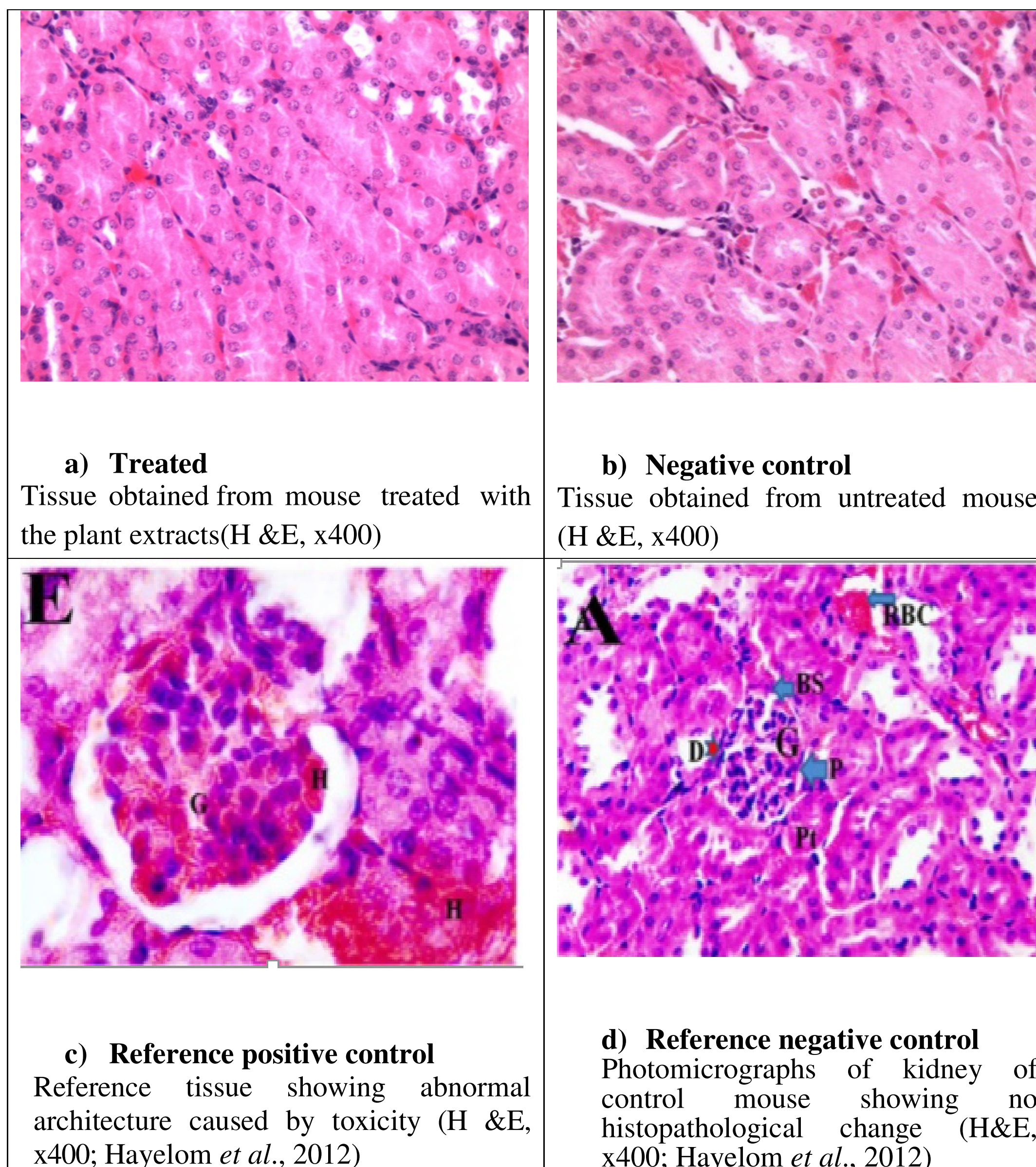
b) Negative control
Tissue obtained from untreated mouse (H &E ,x400)

c) Reference positive control
Reference tissue showing abnormal architecture caused by toxicity (H & E, × 400; Hayelom *et al*., 2012)

d) Reference negative control
Photomicrographs of liver sections A = control mice showing no histopathological change, (H & E, x400; Hayelom *et al*., 2012)

Plate 4.15: Longitudinal section of liver of mouse treated with hexane extracts of *T. diversifolia* flower at 1200 mg/kg.

(a) and (b) shows the histological status of the liver tissues of both the treated and control mice where normal cellular architecture with prominent central vain was shown which indicates, that the extract did not cause damage to livers if used for therapeutic purpose.(c) Positive control indicating inflammations around bile duct and portal vein (I) and vacuolar degenerations (HD) in mice treated at 400mg/kg body weight/day of methanol root extracts of *Clerodendrum myricoides* (d) Normal control, photomicrograph of liver section showing no histological change. Red arrow in positive control indicates endothelial cells. BD = bile duct, CV=Central vein, HD=Hydropic degeneration, I=inflammatory cells, PV=Portal vein.



a) Treated
Tissue obtained from mouse treated with the plant extracts(H &E, x400)

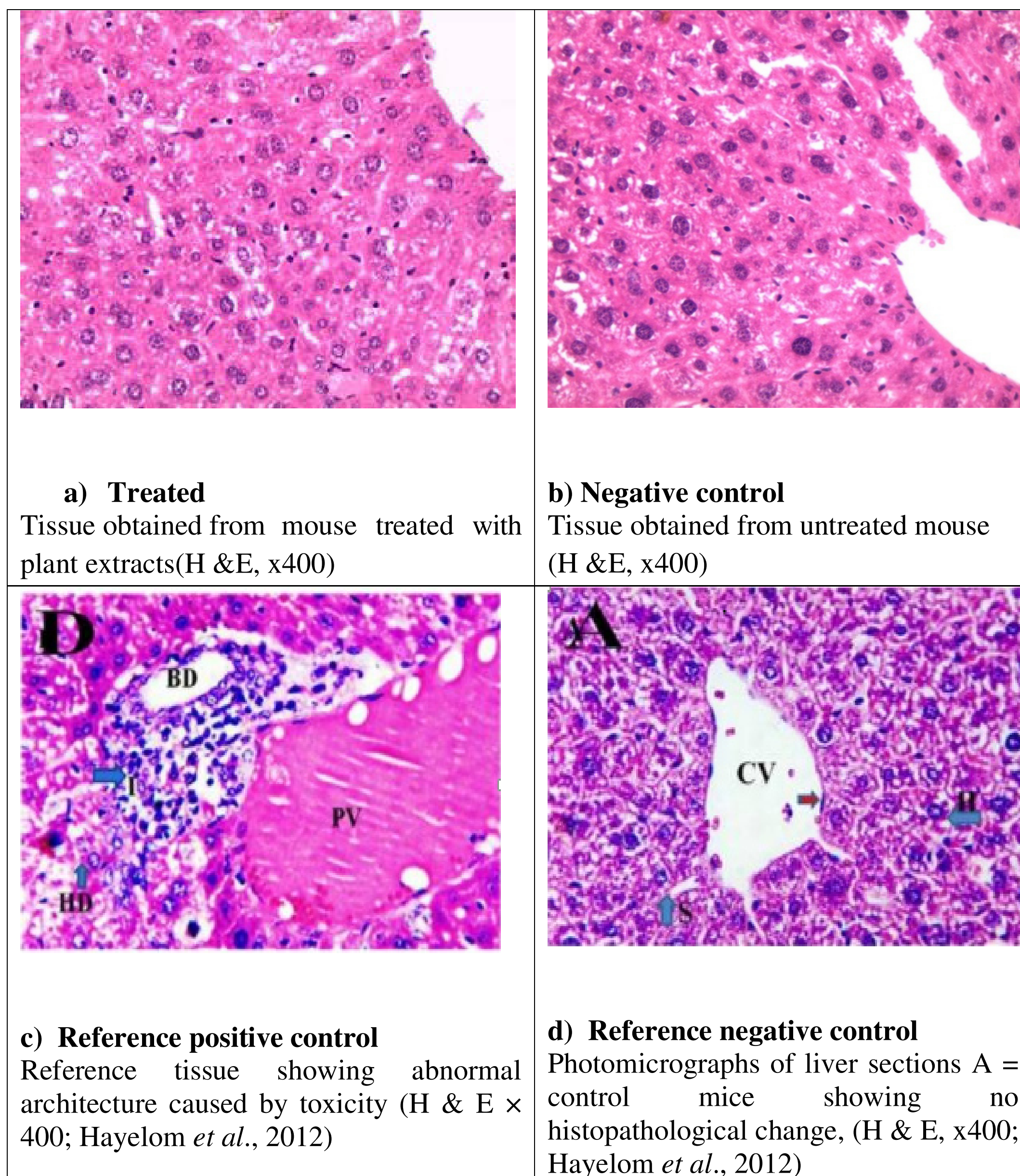
b) Negative control
Tissue obtained from untreated mouse (H &E, x400)

c) Reference positive control
Reference tissue showing abnormal architecture caused by toxicity (H &E, x400; Hayelom *et al.*, 2012)

d) Reference negative control
Photomicrographs of kidney of control mouse showing no histopathological change (H&E, x400; Hayelom *et al.*, 2012)

Plate 4.16: Longitudinal section of Kidney of mouse treated with hexane extracts of *T. diversifolia* flower at 1200 mg/kg.

(a) and (b) are light microscopic sections of the kidney showing no histological changes visible in sections of treated and untreated mice. (c) Positive control Indicating haemorrhage and signs in congestion of the glomeruli of the kidney in mice treated with 1630mg/kg body weight/day of methanol root extract of *Clerodendrum myricoides*. (d) Normal control photomicrographs of kidney indicating there are no histopathological changes visible in the sections of the control mice and red arrow indicates macula densa. D= Distal convoluted tubules, G= Glomeruli, H= Haemorrhage, P= Podocytes, Pt= Proximal convoluted tubules, RBC = Red blood cell.



a) Treated
Tissue obtained from mouse treated with plant extracts(H &E, x400)

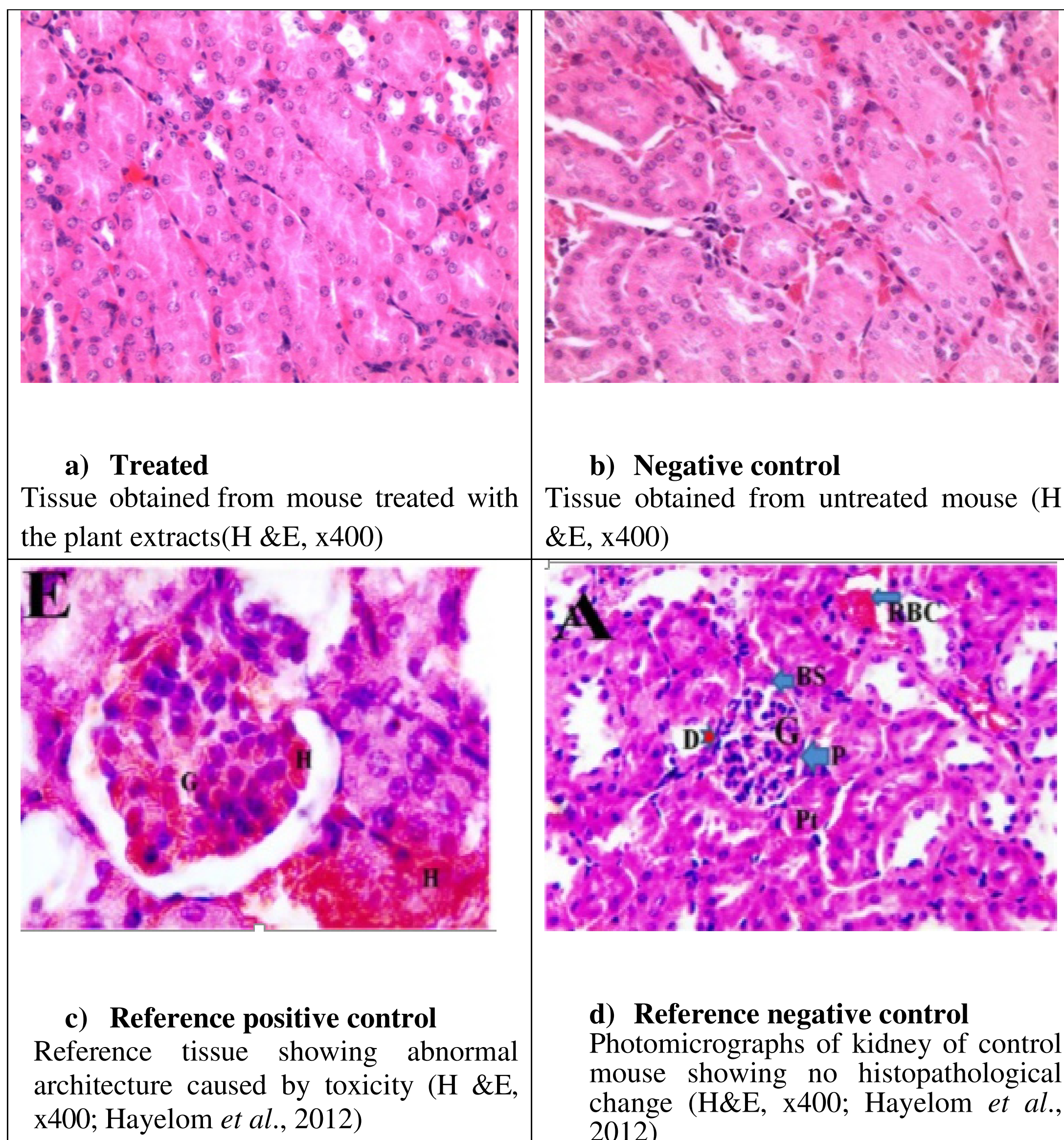
b) Negative control
Tissue obtained from untreated mouse (H &E, x400)

c) Reference positive control
Reference tissue showing abnormal architecture caused by toxicity (H & E × 400; Hayelom *et al*., 2012)

d) Reference negative control
Photomicrographs of liver sections A = control mice showing no histopathological change, (H & E, x400; Hayelom *et al*., 2012)

Plate 4.17: Longitudinal section of liver of mouse treated with ethyl acetate extracts of *W. ugandensis* bark at 1200 mg/kg.

(a) and (b) shows the histological status of the liver tissues of both the treated and control mice where normal cellular architecture with prominent central vain was shown which indicates, that the extract did not cause damage to livers when used for therapeutic purpose.(c) Positive control indicating inflammations around bile duct and portal vein (I) and vacuolar degenerations (HD) in mice treated at 400mg/kg body weight/day of methanol root extracts of *Clerodendrum myricoides* (d) Normal control, photomicrograph of liver section showing no histological change. Red arrow in positive control indicates endothelial cells. BD = bile duct, CV=Central vein, HD=Hydropic degeneration, I=inflammatory cells, PV=Portal vein.



a) Treated
Tissue obtained from mouse treated with the plant extracts(H &E, x400)

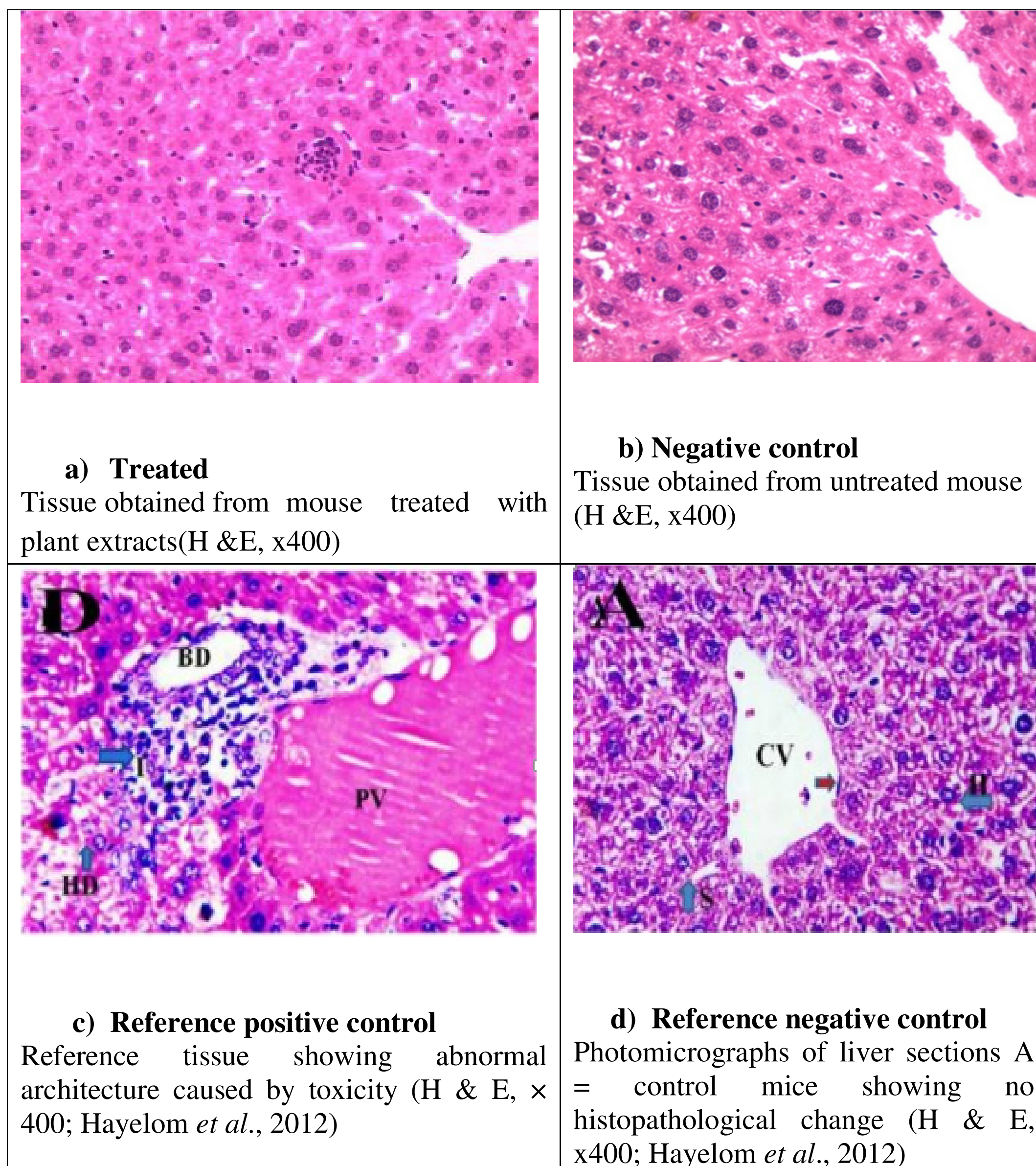
b) Negative control
Tissue obtained from untreated mouse (H &E, x400)

c) Reference positive control
Reference tissue showing abnormal architecture caused by toxicity (H &E, x400; Hayelom *et al*., 2012)

d) Reference negative control
Photomicrographs of kidney of control mouse showing no histopathological change (H&E, x400; Hayelom *et al*., 2012)

Plate 4.18: Longitudinal section of Kidney of mouse treated with ethyl acetate extracts of *W. ugandensis* bark at 1200 mg/kg.

(a) and (b) are light microscopic sections of the kidney showing no histological changes visible in sections of treated and untreated mice. (c) Positive control Indicating haemorrhage and signs in congestion of the glomeruli of the kidney in mice treated with 1630mg/kg body weight/day of methanol root extract of *Clerodendrum myricoides*. (d) Normal control photomicrographs of kidney indicating there are no histopathological changes visible in the sections of the control mice and red arrow indicates macula densa. D= Distal convoluted tubules, G= Glomeruli, H= Haemorrhage, P= Podocytes, Pt= Proximal convoluted tubules, RBC = Red blood cell.



a) Treated
Tissue obtained from mouse treated with plant extracts(H &E, x400)

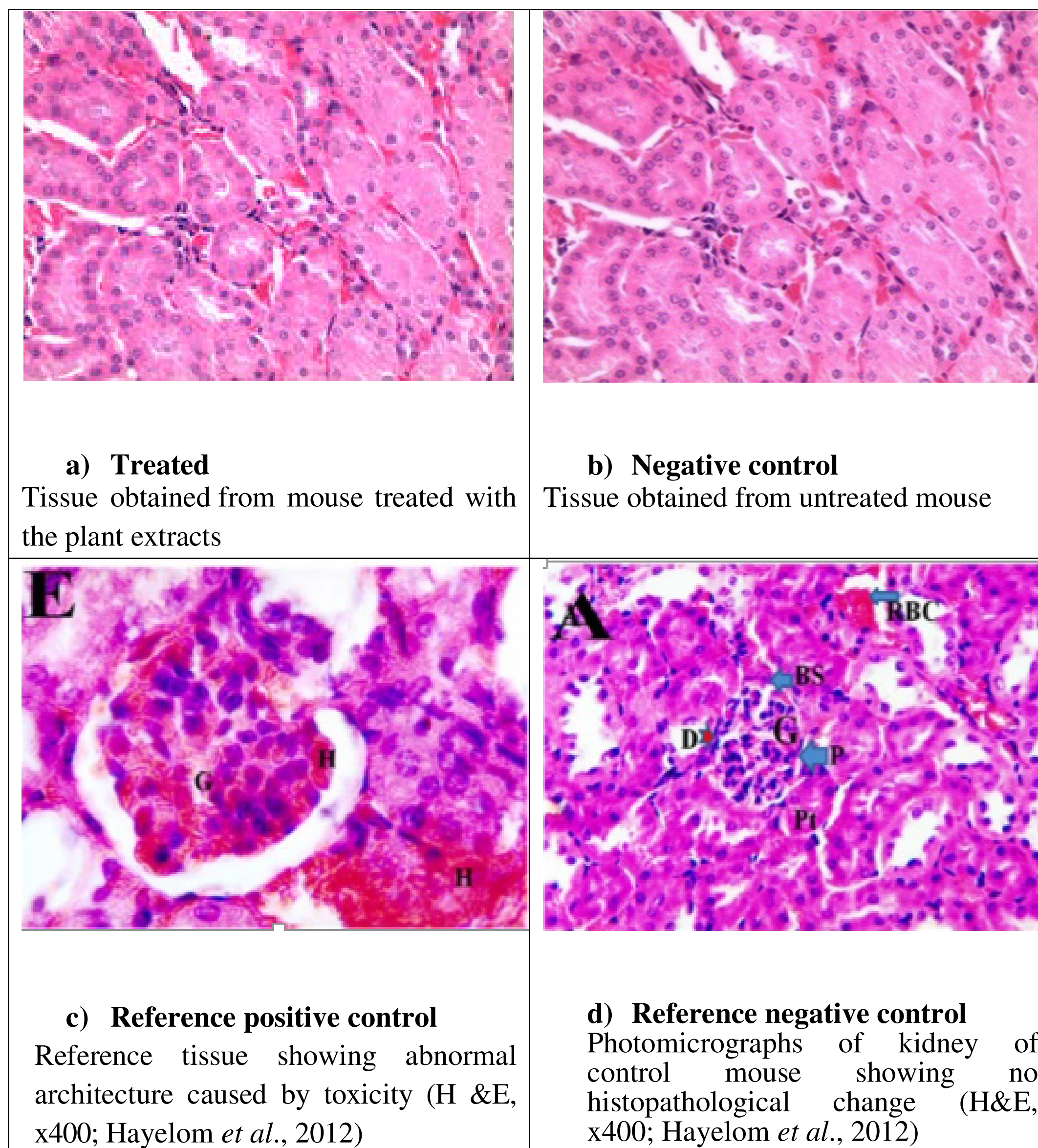
b) Negative control
Tissue obtained from untreated mouse (H &E, x400)

c) Reference positive control
Reference tissue showing abnormal architecture caused by toxicity (H & E, × 400; Hayelom *et al*., 2012)

d) Reference negative control
Photomicrographs of liver sections A = control mice showing no histopathological change (H & E, x400; Hayelom *et al*., 2012)

Plate 4.19: Longitudinal section of liver of mouse treated with hexane extracts of *W. ugandensis* root at 1200 mg/kg.

(a) and (b) shows the histological status of the liver tissues of both the treated and control mice where normal cellular architecture with prominent central vain was shown which indicates, that the extract did not cause damage to livers if used for therapeutic purpose.(c) Positive control indicating inflammations around bile duct and portal vein (I) and vacuolar degenerations (HD) in mice treated at 400mg/kg body weight/day of methanol root extracts of *Clerodendrum myricoides* (d) Normal control, photomicrograph of liver section showing no histological change. Red arrow in positive control indicates endothelial cells. BD = bile duct, CV=Central vein, HD=Hydropic degeneration, I=inflammatory cells, PV=Portal vein.



a) Treated
Tissue obtained from mouse treated with the plant extracts

b) Negative control
Tissue obtained from untreated mouse

c) Reference positive control
Reference tissue showing abnormal architecture caused by toxicity (H &E, x400; Hayelom *et al*., 2012)

d) Reference negative control
Photomicrographs of kidney of control mouse showing no histopathological change (H&E, x400; Hayelom *et al*., 2012)

Plate 4.20: Longitudinal section of Kidney of mouse treated with hexane extracts of *W. ugandensis* root at 1200 mg/kg.

(a) and (b) are light microscopic sections of the kidney showing no histological changes visible in sections of treated and untreated mice. (c) Positive control Indicating haemorrhage and signs in congestion of the glomeruli of the kidney in mice treated with 1630mg/kg body weight/day of methanol root extract of *Clerodendrum myricoides*. (d) Normal control photomicrographs of kidney indicating there are no histopathological changes visible in the sections of the control mice and red arrow indicates macula densa. D= Distal convoluted tubules, G= Glomeruli, H= Haemorrhage, P= Podocytes, Pt= Proximal convoluted tubules, RBC = Red blood cell.

4.5 *In vivo* evaluation of anti-salmonella activity

In the present study four clinical *Salmonella* isolates; *S. ser. Typhi* (ATCC 13347), *S. ser. Typhi* (ATCC 43579), *S. enterica* (ATCC 2162) and *S. ser. Typhimurium* (ATCC 1408) were used for determining *in vitro* anti-salmonella activity. The first three isolates are highly adapted to man and cause typhoid fever. *S. ser. Typhimurium* (ATCC 1408) can infect a wide range of hosts and therefore was used for *in vivo* study of the plant extracts. From the nine plant extracts with *in vitro* anti-salmonella activity, three inhibited the growth *S. ser. Typhimurium* in the mice. Methanol extract of *T. diversifolia* leaf, ethyl acetate and hexane extracts of stem bark and root respectively of *W. ugandensis* tested at three concentrations (300mg/kg, 600mg/kg and 1200mg/kg) showed varied survival rates. Table 4.5 shows plant extracts that were administered orally, twice daily for 5 days at dose of 300, 600 and 1200mg/kg. All mice treated with ethyl acetate extracts of *W. ugandensis* stem bark at 1200mg/kg, survived beyond 30 days. Mice treated with methanol extracts of *T. diversifolia* leaf at 1200mg/kg, showed mean survival time of 25±10.73 (Table 4.5). Mice treated with ethyl acetate extracts of *W. ugandensis* stem bark (600mg/kg) and methanol extracts of *T. diversifolia* leaf (600mg/kg) showed mean survival time of 18±11.42 and 15. ±13.33 respectively.

Table 4.5: ***In vivo*** **anti-salmonella activity of plant extracts**

Extracts/Drug(dose)	Survival time(Days) Mean±SD	No. of mice survive / No. of mice tested	Survival rate (%) after 30 days
TDLM(1200mg/kg)	$25.2±10.73^{ab}$	4/5(30,30,30,30,6)	80
TDLM(600mg/kg)	$15.4±13.33^{cde}$	2/5(5,6,6,30,30)	40
TDLM(300mg/kg)	$4.2±2.05^{g}$	0/5(2,2,5,6,6)	0
TDLE(1200mg/kg)	$10.2±4.03^{defg}$	0/5(6,6,12,12,15)	0
TDLE(600mg/kg)	$6.6±3.05^{fg}$	0/5(5, 5, 5, 6, 12)	0
TDLE(300mg/kg)	$5.6±3.58^{g}$	0/5(1,3,6,9,9)	0
TDFH(1200mg/kg)	$17±11.5^{bcd}$	1/5(5,6,24,24,30)	20
TDFH(600mg/kg)	$9±3.67^{efg}$	0/5(6,6,9,9,15)	0
TDFH(300mg/kg)	$5.6±0.55^{g}$	0(5,5,6,6,6)	0
TDFE(1200mg/kg)	$10.2±4.03^{defg}$	0/5(6,6,12,12,15)	0
TDFE(600mg/kg)	$4.8±0.84^{g}$	0/5(4,4,5,5,6)	0
TDFE(300mg/kg)	$3±1^{g}$	0/5(2,2,3,4,4)	0
TDLH(1200mg/kg)	$5.6±3.58^{g}$	0/5(1,3,6,9,9)	0
TDLH(600mg/kg)	$3.8±1.3^{g}$	0/5(3,3,3,4,6)	0
TDLH(300mg/kg)	$2.6±1.14^{g}$	0/5(1,2,3,3,4)	0
WUSBE(1200mg/kg)	$30±0^{a}$	5/5(30,30,30,30,30)	100
WUSBE(600mg/kg)	18±11.42	2/5(6,9,15,30,30)	40
WUSBE(300mg/kg)	$6±2.74^{g}$	0/5(4,4,4,9,9)	0
WUSBH(1200mg/kg)	$6.2±1.79^{fg}$	0/5(4,6,6,6,9)	0
WUSBH(600mg/kg)	$4±1^{g}$	0/5(3,3,4,5,5)	0
WUSBH(300mg/kg)	$3.4±1.14^{g}$	0/5(2,3,3,4,5)	0
WURE(1200mg/kg)	$6.4±4.88^{fg}$	0/5(3,4,5,15)	0
WURE(600mg/kg)	$3.8±1.1^{g}$	0/5(2,4,4,4,5)	0
WURE(300mg/kg)	$2.6±1.34^{g}$	0/5(1,2,2,4,4)	0
WURH(1200mg/kg)	$20±13.71^{bc}$	3/5(4,6,30,30,30)	60
WURH(600mg/kg)	$17.4±11.7^{bcd}$	2/5(6,9,12,30,30)	40
WURH(300mg/kg)	$8.2±9^{efg}$	0/5(2,4,5,6,24)	0
Cipro(100mg/kg)	$30±0^{a}$	5/5(30,30,30,30,30)	100
DMSO (5%)	$2.8±2.17^{g}$	0/5(1,1,2,4,6)	0

Mean survival time, number of mice survived and % of animal survived after treatment with plant extracts and the controls. The figures in bracket indicate the number of days, each mouse survived before death. TDLM= *Tithonia diversifolia* leaf extract of methanol, TDLE= *Tithonia diversifolia* leaf extract of ethyl acetate, TDFH= *Tithonia diversifolia* flower extract of hexane, TDLH= *Tithonia diversifolia* leaf extract of hexane, TDFE= *Tithonia diversifolia*flower extract of ethyl acetate, WUSBE= *Warburgia ugandensis* stem bark extract of ethyl acetate, WUSBH= *Warburgia ugandensis* stem bark extract of hexane WURE= *Warburgia ugandensis* root extract of ethyl acetate, WURH= *Warburgia ugandensis* root extract of hexane, and DMSO =Dimethyl sulphur dioxide (Negative control), Cipro=Ciprofloxacin, SD= Standard deviation. The means with same superscript letter(s) are not significantly different (P<0.0001).

4.5.1 Body weight of treated mice

Body weight of mice treated with the TDLM plant extract was monitored during the period of the experiment (Figure 4.9). It was observed that the mice treated with 1200mg/kg of methanol extract of *T. diversifolia* leaf showed increase of body weight in the first four days, then was followed by reduction of body weight in day five up to day 8. It was also noted that the body weight of mice increased tremendously from day 9 up to the end of experiment period. Mice treated with 600mg/kg of the plant extract on average maintained their body weight post treatment while the mice treated with 300mg/kg showed reduced body weight. Mice treated with 300mg/kg of the plant extract did not survive beyond day 5. The mice on DMSO (negative control) did not survive beyond 5 days while mice treated with ciprofloxacin (100mg/kg) recorded increase in body weights comparable to plant extract at 1200 and 600mg/kg (Figure 4.9).

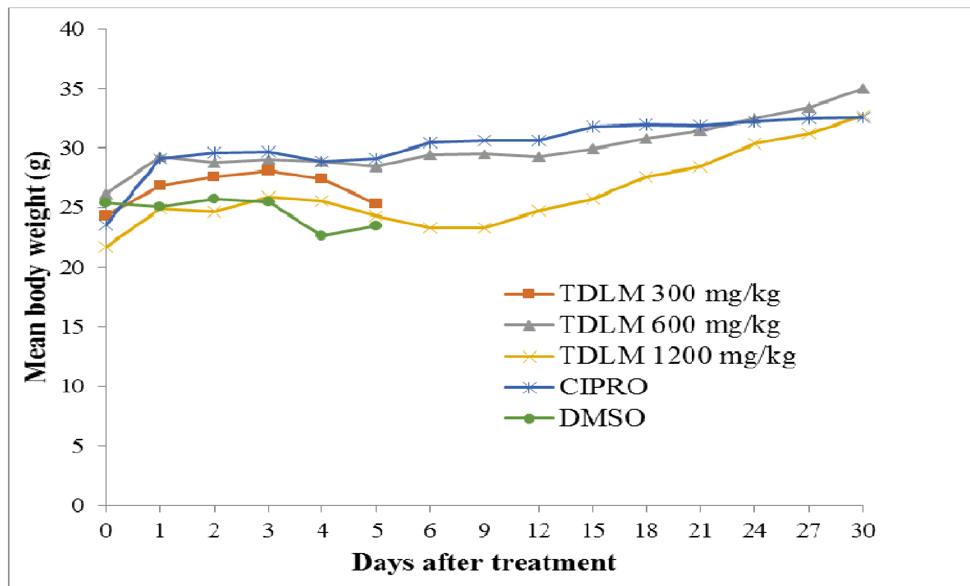


Figure 4.9: Mean body weights (g) of mice treated with TDLM extract

Body weight of mice treated with WUSBE plant extract was monitored during the experiment (Figure 4.10).The mice were treated with three concentrations (300mg/kg, 600mg/kg and 1200 mg/kg) of ethyl acetate extract of *W. ugandensis* stem bark. It was observed that on average mice maintained their body weight post treatment at 600mg/kg and 1200 mg/kg while the mice treated with plant extract at 300 mg/kg showed reduced body weights and did not survive beyond for six days post treatment. For DMSO the mice lost weight and did not survive beyond 5 days post treatment. In contrast, the mice treated with ciprofloxacin increased their body weights until the end of experiment (Figure 4.10).

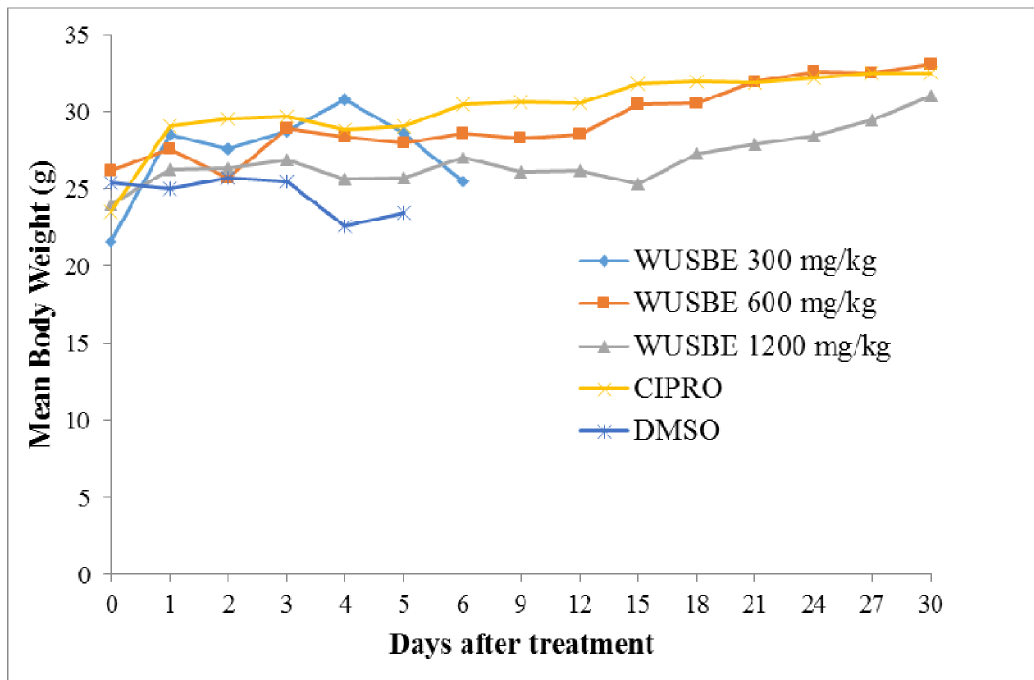


Figure 4.10: Mean body weights (g) of mice treated with WUSBE extract

The mice were treated with hexane extract of *W. ugandensis* at 300mg/kg, 600 mg/kg and 1200mg/kg twice day for 5 days. It was observed that mice treated with 600 mg/kg and 1200mg/kg orally gained weight for the entire period of experiment. The mice that were given 300mg/kg of the plant extract recorded steady weight gain for 6 days. Their weights however decreased after day 9 and did not survive beyond 21 days. For DMSO, mice did not survive beyond 5 days post treatment (Figure 4.11). In contrast, the mice treated with ciprofloxacin recorded steady weight gains post treatment.

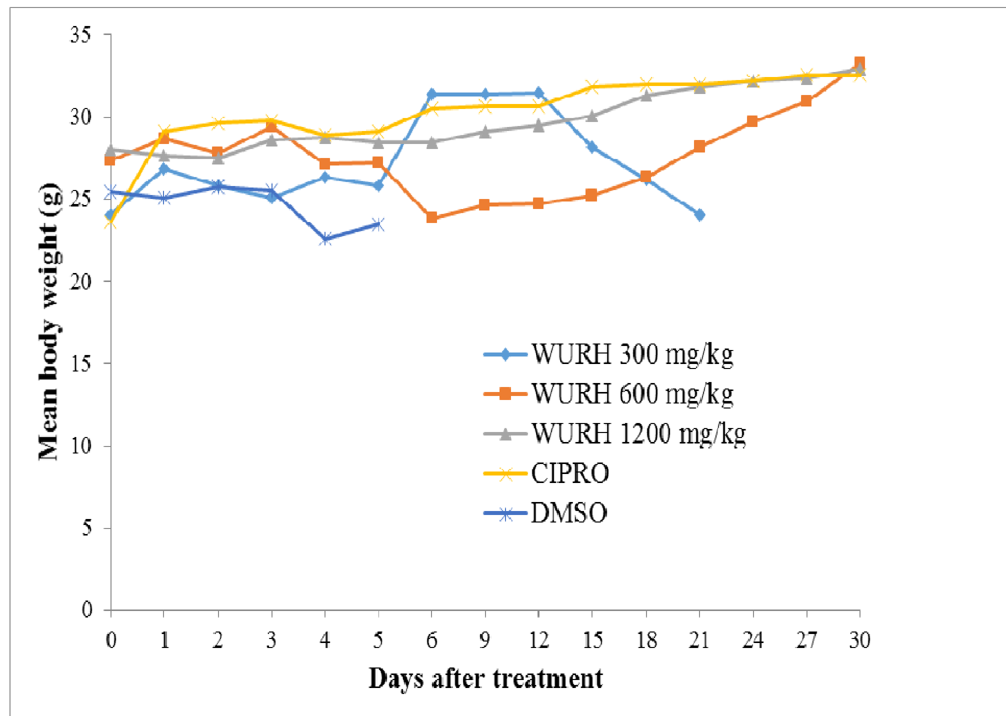


Figure 4.11: Mean body weights (g) of mice treated with WURH extract

4.5.2 *S. ser Typhimurium* in faece shed by mice treated with plant extracts

The mice treated with methanol extract of *T. diversifolia* leaf, ethyl acetate and hexane extracts of stem bark and root of *W. ugandensis* respectively did not demonstrate any growth of *S. ser. Typhimurium* (ATCC 1408) after culturing their feacal shedding in the growth media. The result of faecal shedding of *S. ser. Typhimurium* (ATCC 1408) before and during treatment revealed that all groups did not shed *S. ser. Typhimurium* (ATCC 1408) in their faeces on day 0 (before treatment). Similarly, *S. ser. Typhimurium* (ATCC 1408) was not isolated in faeces samples of mice that were examined throughout the treatment period. However, *S. ser. Typhimurium* (ATCC 1408) was detected in faeces of mice treated with the six plant extracts that did not have activity.

4.6 Identification of compound(s) in active plant extracts

4.6.1 Fractionation of plant extracts by column chromatography

The methanol fractions of *T. diversifolia* leaf were run on TLC and those that gave a similar profile were pooled to give 7 fractions namely T_A, T_B, T_C, T_D, T_E, T_F and T_G. Likewise ethyl acetate fractions of *W. ugandensis* stem bark were pooled to yield 4 fractions namely E_A, E_B, E_C and E_D. Hexane fractions of *W. ugandensis* were pooled to give 7 fractions namely H_A, H_B, H_C, H_D, H_E, H_F and H_G. Resulting 18 column chromatography fractions were evaluated for anti-salmonella activity by microdilution assay.

4.6.2 Anti-salmonella activity of column chromatography fractions

Eighteen column chromatography fractions from methanol extracts of *T. diversifolia* leaf, ethyl acetate stem bark and hexane root extracts of *W. ugandensis* were screened for anti-salmonella activity against 4 clinical isolates of *Salmonella*; *S. ser. Typhi* (ATCC 13347), *S. ser. Typhi* (ATCC 43579), *S. enterica* (ATCC 2162) and *S. ser. Typhimurium* (ATCC 1408) using microdilution assay. Fractions T_A, T_B, T_C, T_D, T_E, T_F and T_G had Minimum inhibitory concentration (MIC) values in the range of 1.22-312.5µg/ml against the 4 clinical *Salmonella* isolates. Meanwhile ciprofloxacin, positive control

had anti-salmonella activity in the range of 1.22-19.53µg/ml. Table 4.6 shows MICs of the seven plant fractions and controls.

Table 4.6: MIC (µg/ml) of methanol fractions of *T. diversifolia* leaf

	Clinical *Salmonella* isolates			
Plant fractions	***S. ser. Typhimurium* (ATCC 1408)**	***S. ser. Typhi* (ATCC 13347)**	***S. ser. Typhi* (ATCC 43579)**	***S. enterica* (ATCC 2162)**
T_A	9.77^{d}	2.44^{d}	4.88^{d}	4.88^{d}
T_B	312.5^{a}	1.22^{d}	19.53^{d}	1.22^{d}
T_C	78.13^{cd}	1.22^{d}	78.13^{d}	1.22^{d}
T_D	39.06^{d}	1.22^{d}	19.53^{d}	39.06^{d}
T_E	39.06^{d}	1.22^{d}	19.53^{d}	39.06^{d}
T_F	39.06^{d}	1.22^{d}	78.13^{cd}	312.5^{a}
T_G	19.53^{d}	4.88^{d}	78.13^{cd}	2.44^{d}
CIPRO	19.53^{d}	1.22^{d}	9.77^{d}	1.22^{d}
Acetone	NA	NA	NA	NA

Values are mean duplicate reading. Mean followed by different superscript letters in the table above are significantly different at P<0.0001. T: *T. diversifolia* leaf, T_A: Combined fractions 1-10, T_B: Combined fractions 11-17, T_C: Combined fractions 18-24, T_D: Combined fractions 25-32, T_E: Combined fractions 33-39, T_F: Combined fractions 40-44 and T_G: Combined fractions 45-50, CIPRO: Ciprofloxacin (Positive control) Acetone: Negative control, NA: No activity.

The four fractions of ethyl acetate extracts of *W. ugandensis* stem bark; E_A, E_B, E_C and E_D had anti-salmonella activity against the 4 clinical isolates of *Salmonella*. Table 4.7 shows anti-salmonella activity values obtained by microdilution assay. The MIC values of fraction E_A and E_B were in the range of 1.22-312.5 µg/ml. Fraction E_C had MIC values of between 1.22 and 156.25 µg/ml while MIC values of fraction E_D ranged between 1.22 and 39.06 µg/ml. Ciprofloxacin standard had MIC values ranging between 1.22 and 19.53 µg/ml against the four clinical isolates.

Table 4.7: MIC (µg/ml) of ethyl acetate fractions of *W. ugandensis* stem bark

Plant Fractions	Clinical *Salmonella* isolates			
	S. ser. Typhimurium **(ATCC 1408)**	***S. ser. Typhi*** **(ATCC 13347)**	***S. ser. Typhi*** **(ATCC 43579)**	***S. enterica*** **(ATCC 2162)**
E_A	156.25[bc]	19.53[d]	156.25[bc]	156.25[bc]
E_B	9.77[d]	2.44[d]	312.5[a]	9.77[d]
E_C	1.22[d]	1.22[d]	19.53[d]	1.22[d]
E_D	1.22[d]	1.22[d]	9.77[d]	1.22[d]
CIPRO	19.53[d]	1.22[d]	9.77[d]	1.22[d]
Acetone	NA	NA	NA	NA

Values are mean duplicate reading. Mean followed by different superscript letters in the table above are significantly different at P<0.0001. E: Ethyl acetate *W. ugandensis* stem bark, E_A: Combined fractions 1-6, E_B: Combined fractions 7-11, E_C: Combined fractions 12-16, E_D: Combined fractions 17-20. CIPRO: Ciprofloxacin (Positive control), Acetone: Negative control, NA: No activity. Values are means of duplicate reading.

Fractions H_A, H_B, H_C, H_D, H_E, H_F and H_G of hexane extracts were also evaluated for anti-salmonella activity by microdilution assay. The MIC values ranged between 1.22 and 312.5 µg/ml. Fraction H_D gave MIC values against the four clinical *Salmonella* isolates which ranged from 1.22 to 19.53 µg/ml. These values were similar to those of ciprofloxacin, (1.22-19.53 µg/ml). Fraction H_F gave MIC values were in the range of 19.53-312.5 µg/ml. Table 4.8 shows MIC values of the seven fractions from *W. ugandensis* root and controls. It is evident from these results that *W. ugandensis* fractions had activity against all the *Salmonella* isolates tested.

Table 4.8: MIC (µg/ml) of fractions of hexane extracts of *W. ugandensis* root

Plant Fractions	Clinical *Salmonella* isolates			
	***S. ser. Typhimurium* (ATCC 1408)**	***S. ser. Typhi* (ATCC 13347)**	***S. ser. Typhi* (ATCC 43579)**	***S. enterica* (ATCC 2162)**
H_A	1.22^{d}	1.22^{d}	156.25^{bc}	312.5^{a}
H_B	1.22^{d}	1.22^{d}	78.13^{cd}	312.5^{a}
H_C	1.22^{d}	1.22^{d}	1.22^{d}	156.25^{bc}
H_D	39.06^{d}	1.22^{d}	1.22^{d}	1.22^{d}
H_E	19.53^{d}	312.5^{a}	156.25^{bc}	312.5^{a}
H_F	78.13^{cd}	1.22^{d}	1.22^{d}	9.77^{d}
H_G	78.13^{cd}	19.53^{d}	4.88^{d}	1.22^{d}
CIPRO	19.53^{d}	1.22^{d}	9.77^{d}	1.22^{d}
Acetone	NA	NA	NA	NA

Values are mean duplicate reading. Mean followed by different superscript letters in the table above are significantly different at P<0.0001. H: Hexane *W. ugandensis* root, H_A: Combined fractions1-16, H_B: Combined fractions 17-23, H_C: Combined fractions 24-39, H_D: Combined fractions 40-58, H_E: Combined fractions 59-69, H_F: Combined fractions 70-89 and H_G: Combined fractions 90-100, CIPRO: Ciprofloxacin (Positive control) Acetone: Negative control, NA: No activity.

4.6.3 Effects of CC fractions on Dihydrofolate reductase (DHFR) activity

4.6.3.1 Extraction and rate of reactions of plant fractions on DHFR

DHFR enzyme was extracted from *S. ser Typhimurium* (ATCC 1408), using the method of Haigh *et al*., (2013). DHFR activity was assayed spectrophotometrically by monitoring the decrease in absorbance at 340nm (due to decrease in NADPH concentration). Eighteen column fractions namely methanol extracts of *T. diversifolia* leaf, ethyl acetate stem bark and hexane extracts of *W. ugandensis* root were screened for their effect on DHFR activity. Trend in change of absorbance at 340nm are shown in Appendix (iv).

4.6.3.2 Effects of plant fractions on *S. ser Typhimurium* dihydrofolate reductase

Eighteen column chromatography fractions from methanol extracts of *T. diversifolia* leaf, ethyl acetate stem bark and hexane extracts of *W. ugandensis* root were screened for their effect on DHFR activity. Methanol fractions namely T_A, T_B, T_C, T_D, T_F and $T_{G,}$ of *T. diversifolia* leaf showed over 83.3% inhibition. All concentrations of fraction T_A, T_B, T_C and T_F, T_D at 512mg/ml, and T_G at 512 and 256mg/ml inhibited the enzyme completely. Fractions T_D (128 and 256mg/ml) and T_G (128 mg/ml) showed the same specific activity (0.2338±0.02µmol/min/mg) demonstrating an inhibitory effect of 83.3% inhibition (Table 4.9). Table 4.9 shows enzyme activity, specific activity, and percentage inhibition of different concentrations of the fractions evaluated. Trimethoprim, inhibited 96.1% of DHFR activity (0.0779±0.12 µmol/min/mg) that was comparable to methanol fractions of *T. diversifolia* leaf.

Table 4.9: Effects of methanol fractions of *T. diversifolia* leaf on DHFR activity

Fractions/ controls	Conc. of fractions/ controls (mg/ml)	Enzyme activity (µmol/min)	Specific activity (µmol/min/mg)	Percent of inhibition
T_A	128	0	0	100
	256	0	0	100
	512	0	0	100
T_B	128	0	0	100
	256	0	0	100
	512	0	0	100
T_C	128	0	0	100
	256	0	0	100
	512	0	0	100
T_D	128	0.5145	0.2338 ± 0.02^{d}	88.3
	256	0.5145	0.2338 ± 0.02^{d}	88.3
	512	0	0	100
T_F	128	0	0	100
	256	0	0	100
	512	0	0	100
T_G	128	0.5145	0.2338 ± 0.02^{d}	88.3
	256	0	0	100
	512	0	0	100
DHFR	2.2	4.4097	2.004	0
TMP	512	0.1715	0.0779 ± 0.12^{f}	96.1
Water		-***	-***	-***
Buffer		-***	-***	-***

-***= Not detected, DHFR activitity=4.4097 µmol/min, Specific activity=2.004 µmol/min/mg. TMP= Trimethoprim (positive control). The means with same superscript letter(s) are not significantly different (P<0.0001). 50mM Buffer (KH_2PO_4) and water were used as blanks.

Table 4-10 illustrates the effect of hexane fractions of *W. ugandensis* root on DHFR activity. All the fractions screened inhibited DHFR activity by more than 41.7%. Fractions H_A (256, 512mg/ml), H_B (512mg/ml), H_E (128,256, 512mg/ml), H_F (512mg/ml) and H_G (128,256, 512mg/ml) inhibited completely DHFR activity. Hexane fractions of *W. ugandensis* root showed mixed inhibitory effect on DHFR activity in the range of 0.1169±0.58 to 1.169±0.3 µmol/min/mg protein. Trimethoprim activity of 0.0779±0.12 µmol/min/mg (96.1% inhibition effect) while specific activity of the DHFR was 2.004 µmol/min/mg.

Table 4.10: Effect of hexane fractions of *W. ugandensis* root on DHFR activity

Fractions /controls	Conc. of fractions/ controls (mg/ml)	Enzyme activity (µmol/min)	Specific activity (µmol/min/mg)	Percent of inhibition
H_A	128	0.5145	0.2338±0.02[d]	88.3
	256	0	0	100
	512	0	0	100
H_B	128	0.2572	0.1169±0.58[e]	94.2
	256	0.5145	0.2338±0.02[d]	88.3
	512	0	0	100
H_D	128	1.0289	0.4677±0.22[c]	76.7
	256	0.5145	0.2338±0.02[d]	88.3
	512	0.5145	0.2338±0.02[d]	88.3
H_E	128	0	0	100
	256	0	0	100
	512	0	0	100
H_F	128	2.5723	1.169±0.3[a]	41.7
	256	1.801	0.8185±0.21[b]	59.2
	512	0	0	100
H_G	128	0	0	100
	256	0	0	100
	512	0	0	100
H_H	128	0.5145	0.2338±0.02[d]	88.3
	256	0.5145	0.2338±0.02[d]	88.3
	512	0.5145	0.2338±0.02[d]	88.3
H_I	128	0.5145	0.2338±0.02[d]	88.3
	256	0.5145	0.2338±0.02[d]	88.3
	512	0.5145	0.2338±0.02[d]	88.3
DHFR	2.2	4.4097	2.004	0
TMP	512	0.1715	0.0779±0.12[f]	96.1
Water		-***	-***	-***
Buffer		-***	-***	-***

Key: -***= Not detected, DHFR activitity= 4.4097 µmol/min, Specific activity= 2.004 µmol/min/mg. TMP= Trimethoprim (positive control). The means specific activity with same superscript letter(s) are not significantly different (P<0.0001). 50mM Buffer (KH_2PO_4) and water were used as blanks.

Ethyl acetate fractions of *W. ugandensis* stem bark namely E_A, E_B, E_C and E_D exhibited DHFR activity (Table 4.11).The inhibitory effect of the 4 fractions ranged from 82.5 to 100%. Fractions E_A (512mg/ml), E_C (512, 256mg/ml) and E_D (512,256, 128 mg/ml) inhibited completely activity of DHFR (Table 4.11). Trimethoprim inhibited DHFR by 96.1% (0.0779±0.12 µmol/min/mg). The activity of DHFR fraction with fraction E_B at three different doses was in range of 0.2338±0.02 to

0.3508±0.12 µmol/min/mg (Table 4.11). Trimethoprim activity specific was 0.0779±0.12 µmol/min/mg (96.1% inhibition effect) while specific activity of the DHFR was 2.004 µmol/min/mg (Table 4.11).

Table 4.11: Effects of ethyl acetate fractions of *W. ugandensis* stem bark on DHFR activity

Fractions/ controls	Conc. of fractions/ controls (mg/ml)	Enzyme activity (µmol/min)	Specific activity (µmol/min/mg)	Percent of inhibition
E_A	128	0.514 5	0.2338±0.02^{d}	88.3
	256	0.5145	0.2338±0.02^{d}	88.3
	512	0	0	100
E_B	128	0.7717	0.3508±0.12^{cd}	82.5
	256	0.6860	0.3118±0.12^{cd}	84.4
	512	0.5145	0.2338±0.02^{d}	88.3
E_C	128	0.5145	0.2338±0.02^{d}	88.3
	256	0	0	100
	512	0	0	100
E_D	128	0	0	100
	256	0	0	100
	512	0	0	100
DHFR	2.2	4.4097	2.004	0
TMP	512	0.1715	0.0779	96.1
Water		-***	-***	-***
Buffer		-***	-***	-***

-***= Not detected, DHFR activitity=4.4097 µmol/min, Specific activity=2.004 µmol/min/mg, TMP= Trimethoprim (positive control). The means specific activity with same superscript letter(s) are not significantly different (P<0.0001). 50mM Buffer (KH_2PO_4) and water were used as blanks.

4.6.4 Phytochemical analysis of CC fractions

A total of eighteen CC fractions from methanol extracts of *T. diversifolia* leaf, ethyl acetate stem bark and hexane root extracts of *W. ugandensis* were screened further to identify the phytochemicals compounds. Phytochemical analysis revealed presence of alkaloids, tannins, flavanoids, steroidal saponin, terpenoidal saponins and glycosides (Table 4.12). Steroidal saponins were detected in 14 out of 18 fractions analyzed and translated to 78% of fractions screened. Terpenoidal saponins were found in 13 fractions out of 18 and this translated to 72% of the fractions analyzed. Glycosides and alkaloids were detected in 11 and 10 out 18 fractions screened and

this occupied 61% and 55% of the fractions analyzed. Tannins were found in two fractions screened. Flavanoids were detected only in fraction E_C (ethyl acetate extract from *W. ugandensis* stem bark). Fractions T_A and T_G (methanol extracts of *T. diversifolia*) showed absence of all the phytochemical compounds tested (Table 4.12).

Table 4.12: Phytochemical constituents of CC fractions

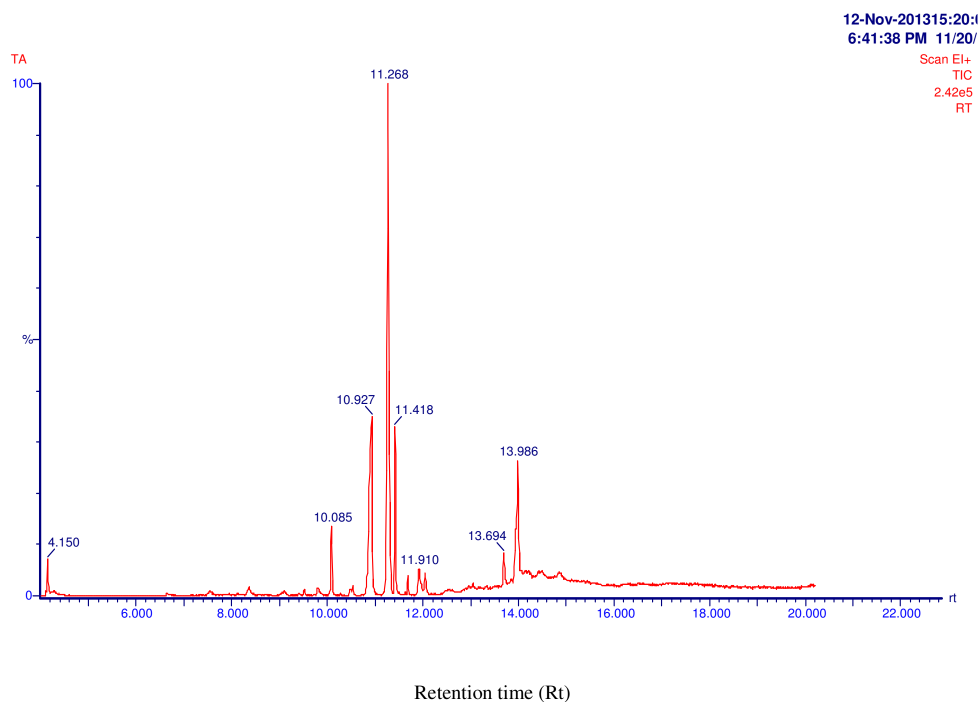
Plant fractions	Alkaloids	Tannins	Flavanoids	Terpenoidal-saponins	Glycosides	Steroidal-saponins
T_A	-	-	-	-	-	-
T_B	+	-	-	-	-	-
T_C	-	-	-	+	+	+
T_D	-	+	-	+	+	+
T_E	+	-	-	+	-	+
T_F	+	+	-	+	-	+
T_G	-	-	-	-	-	-
E_A	+	-	-	++	++	++
E_B	+	-	-	+	+++	+++
E_C	-	-	+	+++	+++	+++
E_D	-	-	-	+	+	+
H_A	+	-	-	+	+	+
H_B	-	-	-	+++	+++	+++
H_C	+	-	-	+	-	+
H_D	+	-	-	+	+++	+++
H_E	+	-	-	+	+	-
H_F	+	-	-	-	+++	+++
H_G	-	-	-	+	+	+

Key -= Absent; **+**= Present; **++**= Moderate concentration and **+++**= High concentration,

4.6.5 Identification of compounds in CC fractions of plants

4.6.5.1 Compounds in TDLM fractions

The seven fractions of methanol extracts from *T. diversifolia* leaf namely T_A, T_B, T_C, T_D, T_E, T_F and T_G were subjected to GC-MS analysis (Appendix vi). The results of T_A, T_B, T_D and T_E are shown in Figure 4.12, 4.13, 4.14 and 4.15 respectively. Fraction T_A showed two compounds, 2, 4-dimethylhexanoic and 3, 4, 5-trimethyl-1-hexene with retention time of 10.927 and 13.694 respectively. Other compounds in fraction T_A were not identified in the NIST library database and need further exploration to reveal identity.

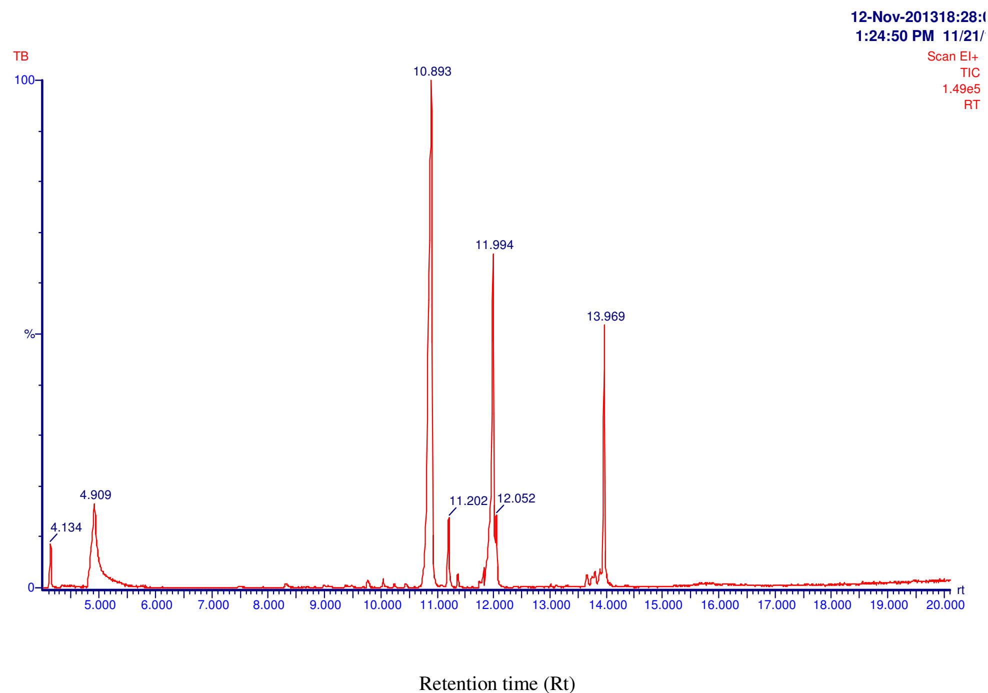


Retention time (Rt)

TA: Fraction T_A of *T.diversifolia*

Figure 4.12: Chromatogram of methanol extract of *T. diversifolia* (T_A)

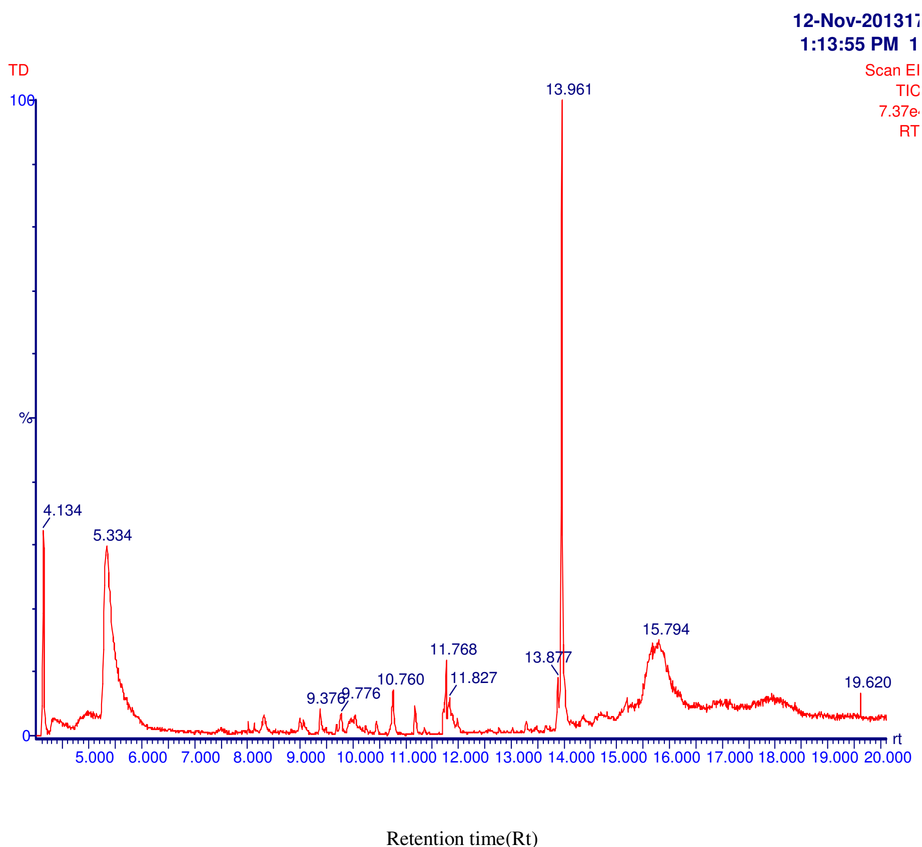
In fraction T_B, n-hexadecanoic acid (palmitic acid) and 9, 12-octadecadienoic acid (Z, Z) were identified with retention times of 10.927 and 11.994 respectively (Figure 4.13). Compounds in fraction T_C with different retention time were not identified in the NIST library database and need further exploration to reveal identity.



TB: Fraction T_B of *T.diversifolia*

Figure 4.13: Chromatogram of methanol extract of *T. diversifolia* (T_B)

Fraction T_D showed two compounds; 1, 2, 3-propanetriolmonoacetate and O-(2-methylpropyl) hydroxylamine with retention time of 5.344 and 9.376 respectively as shown in Figure 4.14.

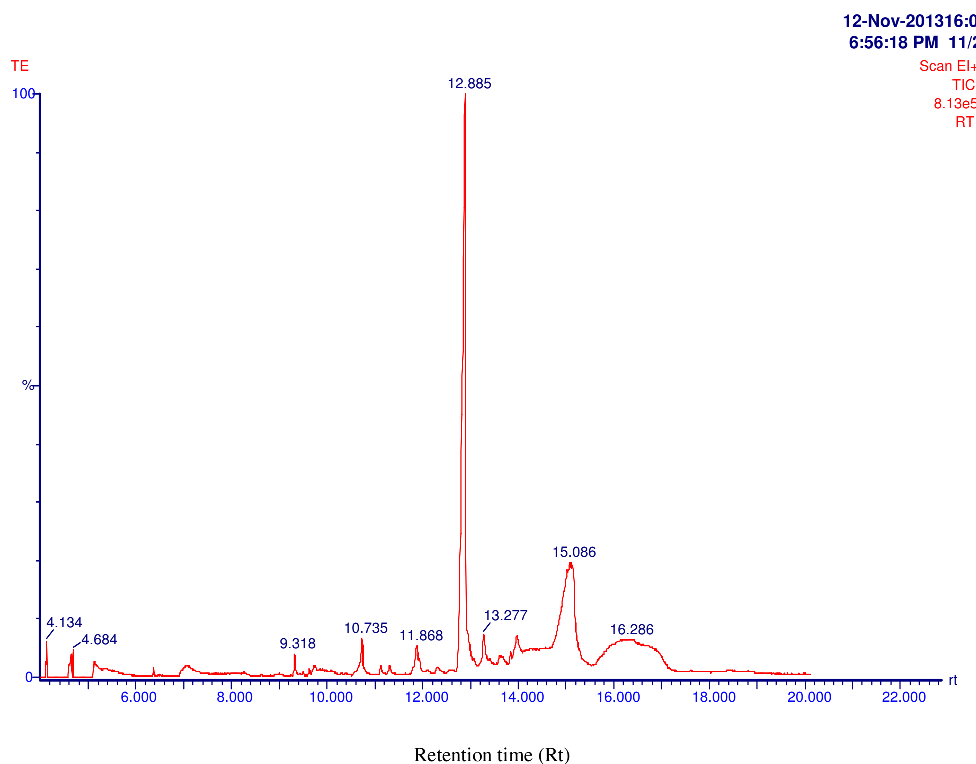


Retention time(Rt)

TD: Fraction T_D of *T.diversifolia*

Figure 4.14: Chromatogram of methanol extract of *T. diversifolia* (T_D)

Fraction T_E showed three compounds; 1, 4:3, 6-dianhydro – D- sorbitol, isorsorbide; E-2-Tetradecen-1-ol and Crotonic acid (o-formylphenylester) with retention time of 4.684, 9.318 and 15.086 respectively as indicated in Figure 4.15. Compounds in fractions T_F and T_G were not identified in the NIST library database and need further exploration to reveal identity.



TE: Fraction T_E of *T.diversifolia*

Figure 4.15: Chromatogram of methanol extract of *T. diversifolia* (T_E)

Table 4.13 illustrates the GC-Ms Retention Time (RT), molecular formula, and molecular weight of individual compounds in fractions from methanol extracts of *T. diversifolia* leaf.

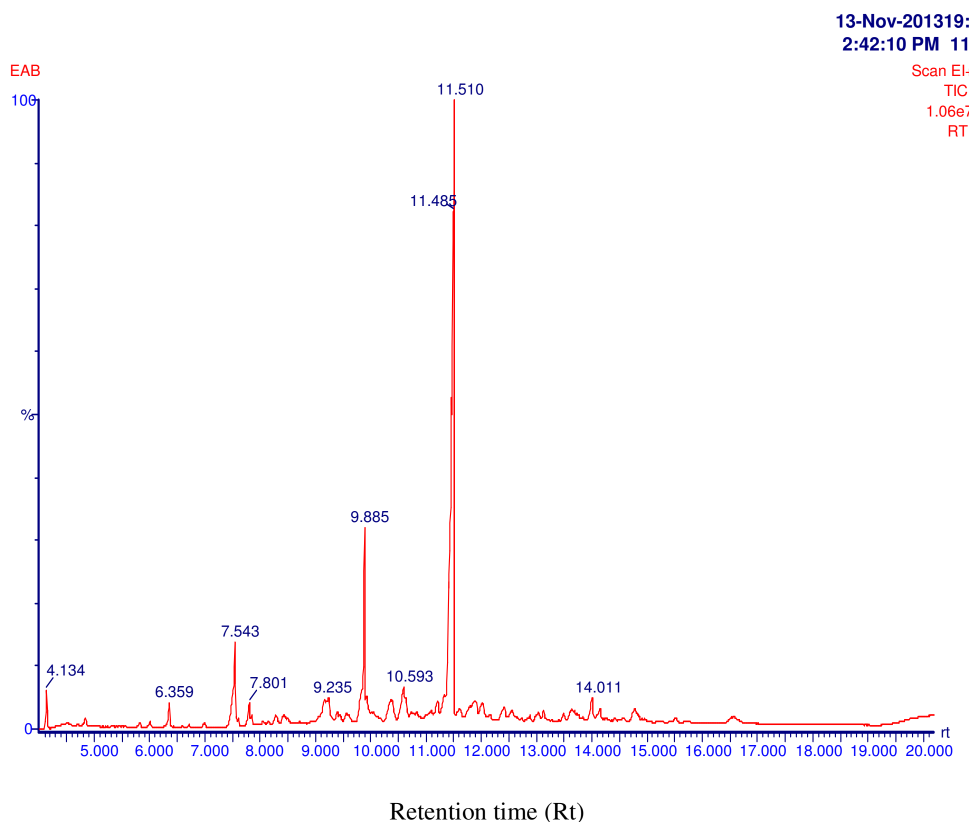
Table 4.13: Compounds identified in TDLM fractions by GC-MS analysis

Fraction	Peaks	$Rt_{(s)}$	M_F	M_W	Name of compound
T_A	1	10.927	$C_8H_{16}O_2$	144	2,4-dimethylhexanoic acid
	2	13.694	C_9H_{18}	126	3,4,5-trimethyl-1-hexene
T_B	1	10.893	$C_{16}H_{32}O_2$	256	n-hexadecanoic acid (palmitic acid)
	2	11.994	$C_{18}H_{32}O_2$	280	
T_C	---	---	---	---	Not determined
T_D	1	5.334	$C_5H_{10}O_4$	134	1,2,3-propanetriolmonoacetate
	2	9.376	$C_4H_{11}NO$		O-(2-methylpropyl)hydroxylamine
T_E	1	4.684	$C_6H_{10}O_4$	146	1,4:3,6-dianhydro – D- sorbitol, isorsorbide
	2	9.318	$C_{14}H_{28}O$	212	E-2-Tetradecen-1-ol,Tetradecanal
	3	15.086	$C_{11}H_{10}O_3$	190	Crotonic acid (o-formylphenylester)
T_F	---	---	---	---	Not determined
T_G	---	---	---	---	Not determined

$Rt_{(s)}$: Retention time (seconds), M_F: Molecular formula and M_W: Molecular weight (grams)

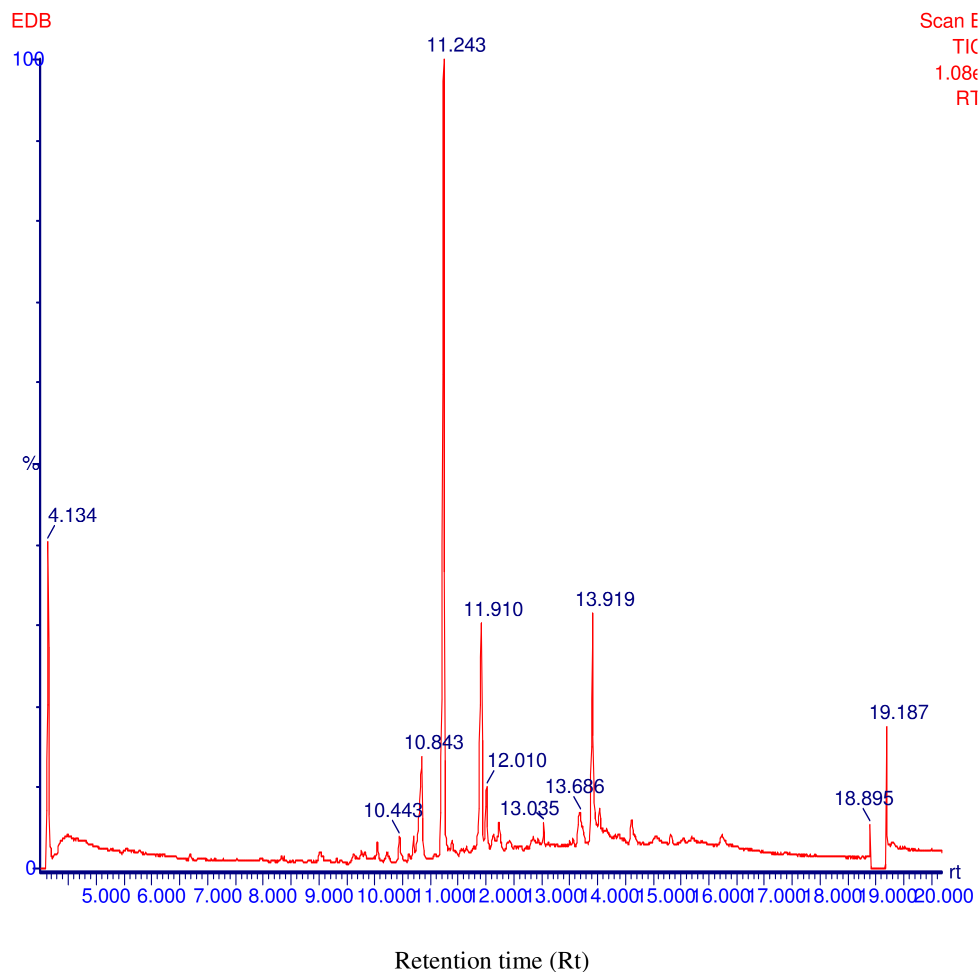
4.6.5.2 Compounds identified in WUSBE fractions

The four fractions from ethyl acetate extract of *W. ugandensis* stem bark (E_A, E_B, E_C and E_D) were subjected to GC-MS analysis and results of fractions E_A and E_D, are shown in Figure 4.16 and 4.17 respectively. Fractions E_A had 6 known compounds and fraction E_D had 2 known compounds (Figure 4.16 and 4.17). The retention time (Rt), molecular formula and molecular weight (MW) of identified compounds are presented in (Table 4-14). Compounds from fractions E_B and E_C were not identified in the NIST library database and need further exploration to reveal identity.



EA: Fraction E_A of ethyl acetate from stem bark

Figure 4.16: Chromatogram of ethyl acetate extract of *W. ugandensis* (E_A)



Retention time (Rt)

EB: Fraction E_D of ethyl acetate from stem bark

Figure 4.17: Chromatogram of ethyl acetate extract of *W. ugandensis* (E_D)

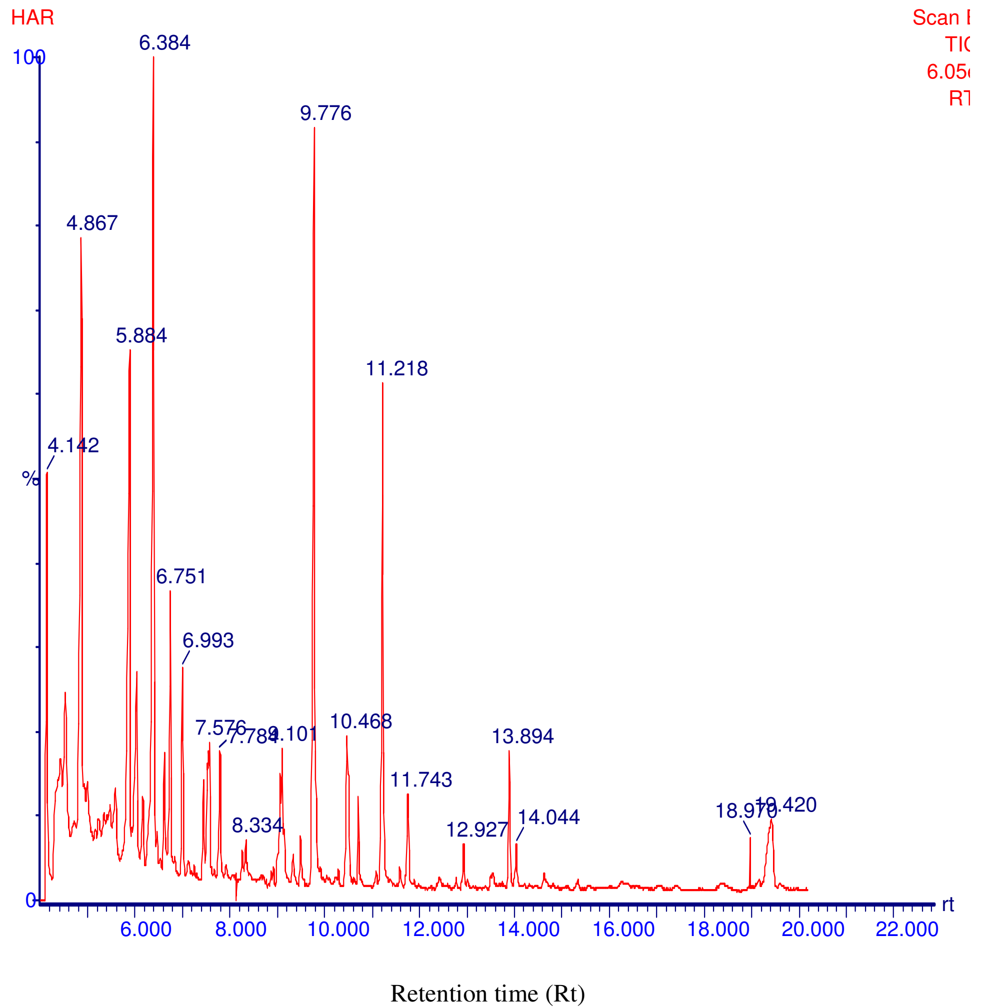
Table 4.14: Compounds identified in WUSBE by GC-MS analysis

Fraction	Peak	$Rt_{(s)}$	M_F	MW	Name of compound
E_A	1	6.359	$C_{13}H_{28}$	184	6-ethyl-2-methyldecane
	2	7.543	$C_{15}H_{24}$	204	Bicyclo[7.2.0]undec-4-ene,4,11,11-trimethyl-8-methylene-
	3	7.801	$C_{15}H_{24}$	204	1,4,8-cycloundecatriene-2,6,6-carophylene
	4	9.235	$C_{15}H_{26}O$	222	1,6,10-Dodecantrien-3-ol,3,7,11-trimethyl-(E)-
	5	10.593	Unknown	Unknown	3-ethenyl-3-methyl-2,1-(1-methylethenyl)-6-(1-methylethyl)cyclohexanol
	6	14.011	$C_{22}H_{42}O_4$	370	Hexanedioic acid, bis (2-ethylhexyl)ester
E_D	1	10.843	$C_{16}H_{32}O_2$	256	Hexadecanoic acid/Palmitic acid
	2	11.910	$C_{10}H_{10}O_4$	252	E-15-heptadecanal

$Rt_{(s)}$: Retention time (seconds), M_F: Molecular formula and M_W: Molecular weight (grams)

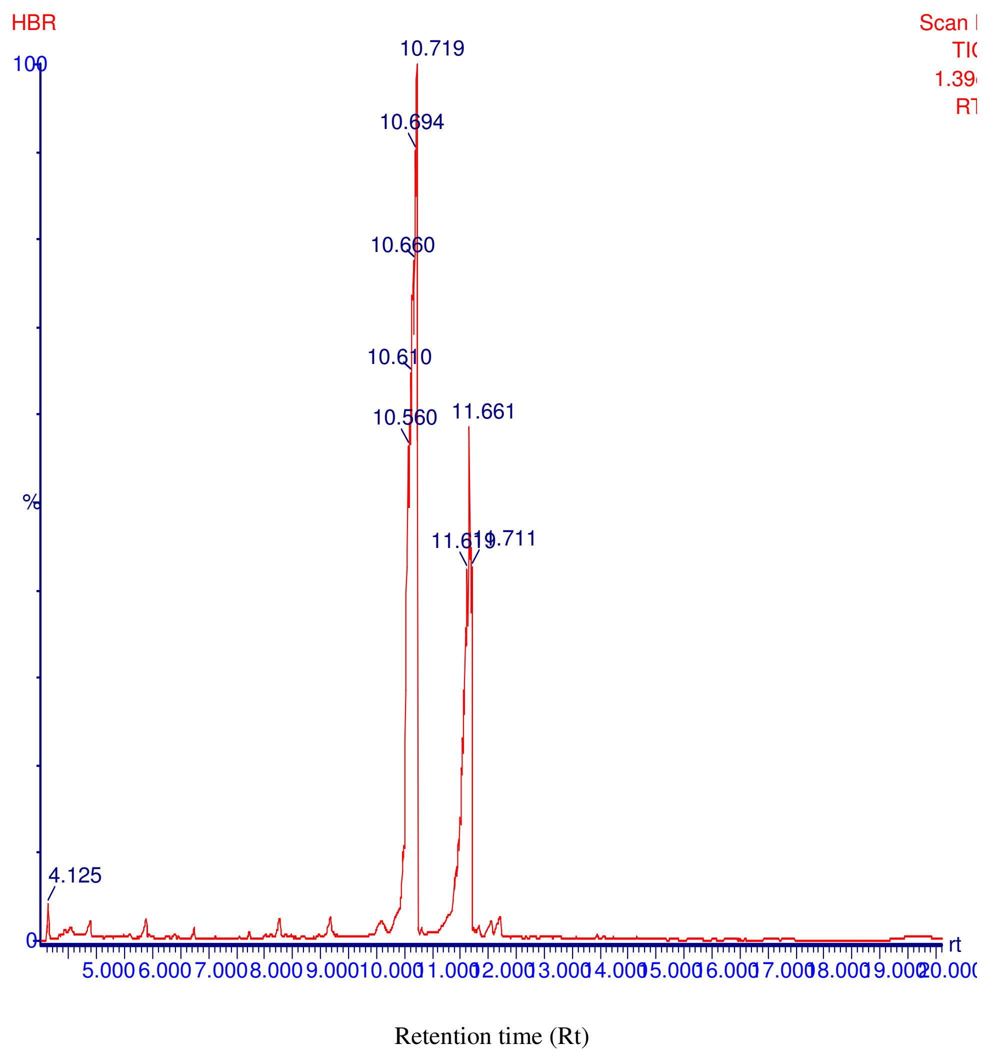
4.6.5.3 Compounds identified in WURH fractions

The seven fractions from hexane extract of *W. ugandensis* root (H_A, H_B, H_C, H_D, H_E, H_F and H_G) were subjected to GC-MS analysis and results of fractions H_A, H_B and H_F, are shown Figure 4.18, 4.19 and 4.20 respectively. Fractions H_A, H_B and H_F had 7, 6 and 3 known compounds respectively (Figure 4.18, 4.19 and 4.20). The compounds with their retention time (Rt), molecular formula (MF) and molecular weight (MW) are presented in (Table 4.15). Compounds from fractions; H_C, H_D, H_E and H_G were not identified in the NIST library database and need further exploration to reveal identity.



HA: Fraction H_A of hexane from root

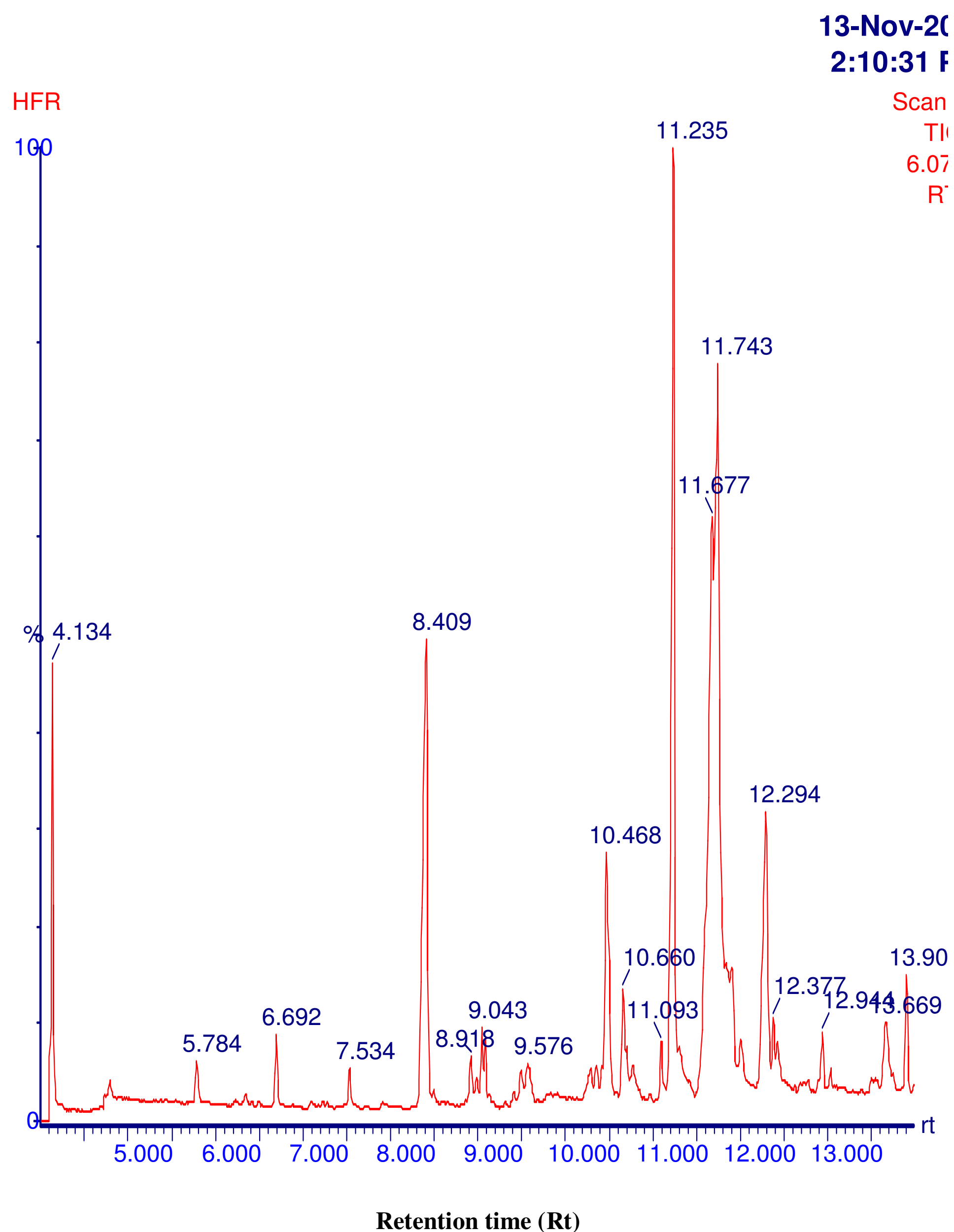
Figure 3.18: Chromatogram of hexane extract *W. ugandensis* (H_A)



Retention time (Rt)

HB: Fraction H_B of hexane from root

Figure 4.19: Chromatogram of hexane extract of *W. ugandensis* (H_B)



Retention time (Rt)

HF: Fraction H_F of hexane from root

Figure 4.20: Chromatogram of hexane extract of W. ugandensis (HF)

Table 4.15: Compounds identified in WURH fractions by GC-MS analysis

Fraction	Peak	$Rt_{(s)}$	M_F	M_W	Name of compound
H_A	1	5.884	$C_{13}H_{28}$	184	6-ethyl-2-methyldecane
	2	6.384	$C_{17}H_{28}O_2$	264	Nerolidyl acetate
	3	6.993	$C_{15}H_{24}$	204	Beta Sesquiphellandrene
	4	9.776	$C_{18}H_{36}O_4$	316	9,10-dihydroxyloctadecanoic acid
	5	11.218	$C_{17}H_{32}O$	252	E-15-heptadecenal
	6	13.894	$C_{24}H_{38}O_4$	390	1,2-benzenedicarboxylic acid, diiisooctyl,
	7	19.410	$C_{13}H_{18}O_2$	206	2-cylohexane-1-one,2,4,4-trimethyl-3-(3-oxo-butenyl)-
H_B	1	10.560	$C_{21}H_{44}$	296	Heptadecane,2,6,10,14-tetramethyl-
	2	10.660	$C_{15}H_{26}O$	242	(S,3E,7E)-α,α,4,8-Tetramethyl-3,7-cyclodecadiene-1-methanol
	3	10.694	$C_{15}H_{26}O$	242	(2,5,5,8a-Tetramethyl-1,4,4a,5,6,8,8a-octahydro-1-napthlenyl)methanol
	4	10.719	$C_{15}H_{24}$	234	Spiro[5,5]undec-2-ene,3,7,7trimethyl-11 methylene (chamigrene)
	5	11.619	$C_{20}H_{32}O_8$	456	Pentacyclo[9.1.0.0(2,4).0(5,7).0(8,10)]dodecane
	6	11.711	$C_{15}H_{22}O_2$	384	(5aS,9aS,9bR)-6,6,9a-Trimethyl-5,5a,6,7,8,9,9a,9b-octahydronaphtho[1,2-c]furan-1(3H)-one (drimenol)
H_F	1	11.677	$C_{17}H_{32}O$	252	E-15-Heptadecenal
	2	12.994	$C_{15}H_{26}O_3$	284	1,1,4,6-Tetramethyldecahydro-1H-cyclopropa[e]azuelene-4,5,6-triol
	3	13.902	$C_{24}H_{38}O_4$	390	1,2-benzenedicarboxylic acid,diisooctyl ester

Rt(s): Retention time (seconds), MF: Molecular formula and MW: Molecular weight (grams).

CHAPTER FIVE

DISCUSSION

5.1 *In vitro* evaluation of anti-salmonella activity

In the present study, the extracts from 5 medicinal plants were screened for antibiotic activity against clinical *Salmonella* isolates. It was noted that of the extracts tested, both ethyl acetate and hexane extracts of *T. diversifolia* and *W. ugendensis* exhibited anti-salmonella activity against the 4 clinical *Salmonella* isolates tested in this study. Methanol extracts of *T. diversifolia* leaf also inhibited the clinical isolates.

Tithonia diversifolia plant extracts exhibited different zones of inhibition against the organisms tested. The ethyl acetate flower and hexane leaf extracts of *T. diversifolia* had zones of inhibition at 18.5±5mm and 17.67±2mm respectively. This compared well with ciprofloxacin that gave zone of inhibition of 19.67mm. There were no significant differences in zones of inhibition exhibited by these extracts and the standard ($p<0.0001$). Methanol extracts of *T. diversifolia* leaf showed significant anti-salmonella activity against the selected clinical *Salmonella* isolates. Moreover, the ethyl acetate and hexane extracts of this plant were found to have slightly higher activity than the methanol extract. The observed anti-salmonella activity of *T. diversifolia* extracts agreed with the finding of Ogunfolakan *et al*., (2010), on broad spectrum antimicrobial activity. This is also supported by Kareru *et al*., (2010), who has reported that soap made from leaf extract of *T. diversifolia* was effective against *E. coli*.

The phytonutrients and secondary metabolites naturally occurring from plants possess antimicrobial activity (Srikumar *et al*., 2007). The anti-salmonella activity observed in this study could be partly attributed to the presence of several metabolic compounds. Phytochemical analysis demonstrated the presence of alkaloids, tannin, flavonoids, terpenoidal saponins, steroidal saponins and glycosides in the active extracts. Among the tested, ethyl acetate flower extracts exhibited the highest anti-salmonella activity. This is due to the differences in the type and concentrations of phytochemical constituents across different plants, variation in anti-salmonella activity are expected. Thus, the

results of this study indicated that the extract had high concentration of steroidal saponins and terpenoidal saponin which could have probably contributed to the inhibition of *Salmonella* growth. This finding agrees with Ashok & Vijayalakshmi, (2013), who demonstrated that sterol from *Vitis* vinifera seed extract exhibited antibacterial activity. The steroidal saponins and terpenoidal saponins could be interacting with the bacterial cell wall and membrane leading to pore formation and degradation of the bacterial component (Devjani & Shah, 2011). Odeyemi *et al*., (2014), has also reported that *T. diversifolia* extracts from leaf, flower and root have antibacterial activity due to the presence of several metabolic compounds such as flavonoids, steroidal saponins and alkaloids.

Ethyl acetate and hexane extract of *W. ugandensis* showed varied zones of inhibition against clinical *Salmonella* isolates tested. Hexane extracts of *W. ugandensis* root and stem bark showed inhibition zones of 14 and 11mm respectively against *S. ser. Typhi* (ATCC 13347). Hexane extract of *W. ugandensis* root and chloramphenicol showed inhibition zone of 10.67mm and 8.67mm against *S. ser. Typhimurium* (ATCC 1408) respectively. However, ciprofloxacin had significantly higher activity at 19.67mm. The observed *W. ugandensis* anti-salmonella activity is supported by Yibeltal *et al*., (2013), who demonstrated activity of crude and semi-purified fractions of *W. ugandensis* against *Shigella boydii* and *Staphylococcous aureus*. Studies carried out by Olila *et al*., (2001), on aqueous extracts of *W. ugandensis* stem bark showed activity against both *Escherischia coli* and *Staphylococcous aureus* in agar well assays but not in the disc diffusion assay. The anti-salmonella activity of *W. ugandensis* observed in the present study could also be attributed to steroidal saponins and terpenoidal saponins, although they occurred in low concentration compared to *T. diversifolia* extracts.

The extracts of three plants namely, *C. megalocarpus, C. endulis* and *L. cornuta* had no anti-salmonella activity as in control. The lack of anti-salmonella activity between extracts and DMSO (Negative control) were not significantly different ($p<0.0001$).

From the 36 plant extracts initially obtained nine were selected because of their appreciable anti-salmonella performance by disc diffusion. The nine extracts were obtained from *W. ugandensis* bark, *W. ugandensis* root, *T. diversifolia* leaf and *T. diversifolia* flower and MIC values determined. The ethyl acetate extract of *W. ugandensis* stem bark demonstrated significant anti-salmonella activity among the extracts tested. The extract had MIC value of 0.031mg/ml against *S. ser. Typhi* (ATCC 13347), *S. ser. Typhi* (ATCC 43579), and *S. ser. Typhimurium* (ATCC 1408). It showed MIC value of 0.061mg/ml against *S. enterica* (ATCC 2162). Hexane extract of *W. ugandensis* root showed MIC value of 0.031mg/ml against clinical *Salmonella* isolates tested except *S. enterica* (ATCC 2162) which was inhibited at 3.91mg/ml. The extracts of hexane stem bark and ethyl acetate root of *W. ugandensis* showed remarkable activity against clinical *Salmonella* isolates tested. Anti-salmonella activity of *W. ugandensis* extracts compared well with ciprofloxacin and chloramphenicol. In a study carried out by Yibeltal *et al*., (2013), on antimicrobial activity of crude extracts of *W. ugandensis* against *E.coli* and *Pseudomonas aeruginosa* demonstrated MIC values of 1.75mg/ml which compared well with result of this study.

The methanol extract of *T. diversifolia* leaf had MIC values of 0.031, 0.24, 0.98 and 0.488 µg/ml against *S. ser. Typhi* (ATCC 43579), *S. ser. Typhi* (ATCC 13347), *S. ser. Typhimurium* (ATCC 1408) and *S. enterica* (ATCC 2162) respectively. Methanol and ethyl acetate extracts of *T. diversifolia* exhibited MIC values of 0.031mg/ml each against *S. ser. Typhi* (ATCC 43579) and *S. enterica (*ATCC 2162) respectively. These values were not significantly different from ethyl acetate extracts of *W. ugandensis* ($p<0.0001$). It was noted in the present study that clinical *Salmonella* isolates were sensitive to all *T. diversifolia* extracts but at different MIC values. This study has demonstrated lower MIC values for *T. diversifolia* extracts against *Salmonella* isolates than what Ogundare, (2007), reported. Ogundare reported MIC values of chloroform and methanol extracts of *T. diversifolia* at 6.25 mg/ml and 3.125 mg/ml respectively against *S. typhi*. The two extracts however showed MIC of 6.25 mg/ml each against *Pseudomonas aeruginosa*.

It was noted from the present results that plant extracts tested by microdilution technique showed reliable anti-salmonella activity as compared to disc diffusion technique. *Warburgia ugandensis* extracts showed lower MIC values when determined by microdilution method than by disc diffusion method. In most cases the activity observed by disc diffusion is not evident when determined by microdilution technique. *Warburgia ugandensis* stem bark had activity of 6±0 mm compared to 0.031µg/ml against S. ser. Typhi (ATCC 13347). This is supported by Olila *et al*., (2001), who reported that the paper disc retains the active component and does not allow it to diffuse into Muller Hinton agar. The paper disc is composed of cellulose [b-(1-4) linked glucose monomers]. The many free hydroxyls groups present on each glucose residues renders the surface hydrophilic (Burgess *et al*., 1999). Thus, if natural products were cationic, they would be expected to adsorb to the surface of the disc and not diffuse into the medium. Consequently, a cationic polar compound displays a good antibacterial activity, but which is therefore not noticeably antibacterial by paper disc diffusion (Cleidson *et al*., 2007).

Most of the antibiotics used nowadays have lost their effectiveness due to development of resistance strains of microbes, which is primarily due to expression of resistance genes (Davies, 1994; Service, 1995). The antibiotics are sometimes associated with side effects such as hypersensitivity, immune suppression and allergic reaction (Ahmad *et al*., 1998). Therefore, more interest is shown to develop alternative antimicrobial drugs for the treatment of infectious diseases without side effects (Berahou *et al*., 2007; Salomao *et al*., 2008). The results of the present study demonstrate anti-salmonella activity of *W. ugandensis* and *T. diversifolia* that compared well with ciprofloxacin and chloramphenicol. This is most encouraging among the 9 active plant extracts tested.

5.2 Acute and sub chronic toxicity studies

Herbal medicines are widely perceived by the public as being natural, therapeutic and free from side effects. Most people believe that herbal medicines have no side effects due to their natural origin. They are often considered as food supplements and

not drugs. Medicinal herbs are usually self-prescribed by the consumers and there is a lack of control and review in terms of dose, manner, and frequency of administration. The chemicals in medicinal herbs may be natural to the plant, but they are not natural to the human body. Any compound with therapeutic effect has the potential to cause harm if incorrectly prescribed or overdosed. This study focused on plants which have been extensively used among the Abagusii community in Kenya for their medicinal properties. The increased use of these plants have resulted in concerns over both their efficacy and safety.

A good drug is one that distributes to all potential sites of infection in the body. If toxic, the drug will exert its adverse effect on multiple tissues and body organs. This is especially true for the liver and the kidney, the main sites of metabolism and drug excretion respectively. The idea behind the oral administration of the plant extracts in this study to mice was that if the doses prescribed by traditional practitioners are not toxic to patients, they should not be toxic to mice, under the same conditions and may even be active. In acute toxicity studies; the Swiss white mice were treated with methanol, ethyl acetate and hexane extracts of *T. diversifolia* and *W. ugandensis* single dose orally (2000mg/kg). There were no mortalities or signs of toxicity recorded. Mice had normal weight gain and healthy. The PCV was in the range of 42.2-50.6%. This is comparable to references value of 42-52%. Therefore there were no significant differences in mean body weight, packed cell volumes and general behaviors of mice given plant extracts in a single dose($p<0.0001$). These results indicate no toxicity effect of the plant extracts. Histopathological examinations of the liver and the kidney did not reveal any morphological changes after administration of the plant extracts. Treated mice showed no liver inflammation around bile duct and portal vein and vacuolar degenerations at doses of 2000 mg/ml. Kidney haemorrhage and congestion of the glomeruli was also not observed. The results suggest that the methanol extract of *T. diversifolia* leaf, ethyl acetate and hexane extracts from *W. ugandensis* stem bark and root respectively are not toxic to mice after acute exposure. The present study agrees with what Karani *et al*. (2013), reported on the safety and efficacy of *Prunus africana* and *Warburgia ugandensis* against induced Asthma in BALB/c Mice. They demonstrated that on administration of single doses of varied

concentrations of *W. ugandensis* to mice, no mortality was observed even at the highest concentration and the LD50 was >5000mg/kg body weight. Based on the scale of Loomis and Hayes (Karani *et al*., 2013) on classification of toxicity, *W. ugandensis* was classified as relatively harmless with LD50 >5000 mg/kg body weight. Another pointer to the safety of the extracts tested was that all the animals that received the extracts stayed alive for the entire period of study and the progressive weight was not significantly different from that of controls. In addition, the present findings of acute toxicity of methanol extracts *T. diverfolia* leaf is in line with Ezeonwumelu *et al*., (2012), who previously reported on acute toxicity and anti-diarrhoeal effect of aqueous extract of Kenyan *T. diversifolia* leaves in rats. According to him treating rats with aqueous extracts *T. diversifolia* established an LD_{50} ranging between 5000mg/kg to 10000mg/kg body weight. This was regarded generally safe and remotely intoxicating.

The high degree of safety is also consistent with its popular use locally. Moreover Claudia *et al*., (2015), has also provided evidence that is in line with present finding that *T. diversifolia* (Hemsl.) A. Gray exhibits health promoting properties, due to its free radical scavenger capacity. This protects cellular systems involved in stress management for example adipogenesis i.e. mesenchymal cells.

In sub-chronic toxicity study, male Swiss white mice were treated with *T. diversifolia* and *W. ugandensis* extracts at dose of 300, 600 and 1200mg/kg/ body weight administered orally for period of 30 days. It was observed the mice exhibited normal behavior and respiratory pattern. The skin and fur was normal. The PCV and mean body weights were normal. No death was recorded in the period of 30 days (100% survival). Histological status of the liver and kidney tissues of both treated and control mice were not damaged. Cells and central vein appeared normal to liver and kidney. The integrity of liver and kidney tissues was not adversely affected at doses of 300, 600 and 1200 mg/ml compared to the control after 30 days of experiment. This indicates that the stem bark and root extracts of *W. ugandensis* were safe to use in mice as herbal medicine at the doses tested. The present investigation concur with previous studies that indicate that all parts of *W. ugandensis* are edible and that the leaves, barks, young shoots and fruits are used as food and as medicine (Karani *et al*., 2013). Besides, *W. ugandensis* bark extracts

have been used for treatment of stomach worms and malaria in Baringo Kenya (Karani *et al*., 2013). They have also been observed to be non-toxic to BALB/c macrophages (Githinji *et al*., 2010).

This study has shown that the nine extracts from *T. diversifolia* and *W. ugandensis* namely TDLE, TDFH, TDLM, TDLH, TDFE, WURE, WURH, WUSBE and WUSBH have *In vitro* anti-salmonella activity. They are also non toxic to key organs like liver and kidney. To assess further their anti-salmonella potential in vivo studies were conducted using Swiss white mice.

5.3 *In vivo* anti-salmonella activity

The results of *in vivo* studies showed that only three of the nine extracts tested were able to inhibit growth *S. ser. Typhimurium* in the mice. Methanol extract of *T. diversifolia* leaf, ethyl acetate and hexane extracts of stem bark and root of *W. ugandensis* tested at different concentrations were effective, with survival rate in the range of 40-100%. The extracts of *W. ugandensis* stem bark (ethyl acetate) at 600mg/kg and 1200 mg/kg had survival rate of 40 and 100% respectively. Likewise *T. diversifolia* leaf (methanol) showed survival rate of 40 and 80% respectively. Moreover *W. ugandensis* root (hexane) tested at 600 and 1200mg/kg indicated survival rate of 40 and 60% of mice treated. The treatment was administered 24 hours post- infection. There was no *S. ser. Typhimurium* found in feaces after culturing. It was observed based on survival rating that ethyl acetate extract of *W. ugandensis* stem bark was the best against *S. ser. Typhimurium* (ATCC 1408) closely followed by methanol extract of *T. diversifolia* leaf. In addition, *W. ugandensis* root (hexane) was the least among the three extracts tested.

Warburgia ugandensis stem bark extract was evaluated at concentrations; 300mg/kg, 600mg/kg and 1200 mg/kg. The survival times were 6 days, 18 days and 30 days respectively compared to 2.8 days of animals treated with DMSO (control). Ciprofloxacin treatment registered survive time of 30 days. This compared well with survival times of mice treated with *W. ugandensis* stem bark (ethyl acetate) 1200mg/kg body weight ($p<0.0001$). Mice treated with *T. diversifolia* leaf extract at concentrations; 300mg/kg, 600mg/kg and 1200 mg/kg

exhibited survival times of 4.2 days, 15.4 days and 25.2 days respectively compared to 2.8 days of animals treated with DMSO (control). Likewise, mice treated with *W. ugandensis* root extract at 300mg/kg, 600 mg/kg and 1200mg/kg exhibited survival times of 8.2 days, 17.4 days and 20 days respectively. Of the doses tested *W. ugandensis* stem bark, *T. diversifolia* leaf and *W. ugandensis* root, 1200 mg/kg was most effective with survival rate of 100%, 80% and 60% respectively. All the mice survived had a normal weight gains and no apparent ill effects.

The body weight change serves as a sensitive indicator of the general health status of animals and it is significant if the body weight loss that occurred is more than 10% from the initial weight (Nweke *et al*., 2012). Hence, the insignificant difference observed in body weight of mice given extracts in this study indicates that the three extracts; *W. ugandensis* stem bark, *T. diversifolia* leaf and *W. ugandensis* root, had no apparent adverse effect on the health of the mice.

W. ugandensis stem bark, *T. diversifolia* leaf and *W. ugandensis* root extracts used in this study exhibited significant antimicrobial activity against *S. ser. Typhimurium*. They reduced bacteria in the faeces to undetectable levels. This finding suggests that all the plants used in this experiment were effective against the test organism *in vivo* having almost the same potency. The differences in survival rate exhibited by the plant extracts in reducing the concentration of *S. ser. Typhimurium* in the faeces could be due to the type and the quantity of bioactive ingredients present in the plants (Gricilda & Molly, 2001).

Most studies on biological activities of *W. ugandensis* and *T. diversifolia* extracts and constituents have been connected to traditional uses. *Warburgia ugandensis* (Canellaceae) is a medicinal plant, traditionally used as herbal medicine for a wide range of diseases in some parts of Ethiopia (Wube *et al*., 2010). The traditional medicinal practitioners use *W. ugandensis* to treat malaria, tuberculosis bronchitis, pneumonia, hepatitis, tapeworm, gonorrhea, and asthma in Dollo Menna, Baleregion of Ethiopia (Wube *et al*., 2010). Most of the biological activities are attributed to the drimane sesquiterpenoids, including polygodial, warburganal, muzigadial, mukaadial and ugandensial, flavonoids and

miscellaneous compounds present in the various species (Leonard *et al*., 2015). This agrees with the observed anti-salmonella activity and phytochemical analysis of *W. ugandensis* extracts in the present study.

Tithonia diversifolia (Hemsley) A. Gray commonly known as Mexican sunflower or Mexican arnica is a member of the family Asteraceae. It is an annual weed growing aggressively in abandoned lands, road-sides, river banks and cultivated farmlands. The plant is adaptable to most soils (Olabode *et al*., 2007). Though a native of Central America, it has become naturalized in many tropical countries including the North-East regions of India. In Manipur the plant is widely grown in wild, especially at the foothills and roadsides. There had been reports of *Tithonia* being used for a wide variety of purposes including medicinal and ethnobotanical values (Garcia & Delgado, 2006). Scientific research has established that skin care products formulated from *T. diversifolia* extracts had remarkable antimicrobial properties (Kareru *et al*., 2010). It has been reported that dichloromethane leaf and flower extracts of *T. diversifolia* have antiplasmodial (Muganga *et al*., 2010). In general, the genus *Tithonia* is an important source of diverse natural products, particularly sesquiterpene lactones, diterpenes, and flavonoids. They are known to possess wide variety of biological and pharmacological activities such as antimicrobial, cytotoxic, anti-inflammatory, antiviral, antibacterial, antifungal activities, effects on the central nervous and cardiovascular systems as well as allergenic potency (Chagas-Paula *et al*., 2012). Their wide structural diversity and potential biological activities have rekindled further interest among chemists (Chaturvedi, 2011).

The ethyl acetate extract of *W. ugandensis* stem bark, methanol extract of *T. diversifolia* and hexane extract of *W. ugandensis* root showed no toxicity when administered to the mice and so these studies provided further evidence of the safety and efficacy against typhoid fever. Moreover, the three extracts of the two medicinal plants were re-evaluated further and this was done by bio-guided fractionations, isolation and identification of major compounds in the extracts.

5.4 Anti-salmonella activity of CC fractions of plant extracts

Methanol extracts of *T. diversifolia* leaf, ethyl acetate extracts of *W. ugandensis* bark and hexane extracts of *W. ugandensis* root were fractionated separately by column chromatography technique. Seven methanol fractions of *T. diversifolia* leaf designated T_A, T_B, T_C, T_D, T_E, T_F and T_G were obtained after separation. Likewise four ethyl acetate fractions of *W. ugandensis* bark designated E_A, E_B, E_C and E_D were obtained and seven hexane fractions of *W. ugandensis* root designated H_A, H_B, H_C, H_D, H_E, H_F and H_G were also obtained. All fractions were evaluated for anti-salmonella activity by microdilution assay.

The MICs values of the seven fractions of methanol extract of *T. diversifolia*; T_A, T_B, T_C, T_D, T_E, T_F and T_G were in the range of 1.22-312.5µgml^{-1} against the 4 clinical *Salmonella* isolates. The MIC values of T_A were 9.77, 2.44, 4.88 and 4.88 µg/ml against *S. ser. Typhimurium* (ATCC 1408), *S. ser. Typhi* (ATCC 13347), *S. ser Typhi* (ATCC 43579), *S. enterica* (ATCC 2162) respectively. The MIC value as low as 2.44µg/ml of a column chromatography fraction against the clinical *Salmonella* isolates is suggestive of good anti-salmonella activity of the compound T_A. The MIC values of T_B were in the range of 1.22-312.5µg/ml against the four isolates tested. The lowest MIC value noted for this fraction was 1.22 µg/ml against *S. ser. Typhi* (ATCC 13347) and *S. enterica* (ATCC 2162) where as *S. ser. Typhimurium* (ATCC 1408) was the least sensitive with MIC value of 312.5 µg/ml. The MIC values for fraction T_C, T_D, T_E, T_F and T_G were in the range of 1.22-312.5 µg/ml. It was noted in present study that clinical *Salmonella* isolates were sensitive to all methanol *T. diversifolia* fractions at different MIC values. This compared well with ciprofloxacin which gave MIC values of 1.22 to 19.53µg/ml and there was no significant difference in the activity observed ($P<0.0001$). The observed anti-*Salmonella* activity of *T. diversifolia* fractions agrees with the finding of Obafemi *et al*., (2006), on broad spectrum antimicrobial activity on germacranolide type sesquiterpene lactone from *T. diversifolia* leaf extract (MICs = 15.6 – 62.5mg/ml for most strains of bacteria tested). Meffo *et al*., (2006), has also reported that tithoniaquinone A isolated from leaves of *T. diversifolia* showed strong antibacterial activity against the Gram-positive bacterium *Bacillus*

megaterium and antifungal activity against *Microbotryum violaceum.*

The phytochemicals and secondary metabolites from plants possess antimicrobial activity (Srikumar *et al*., 2007). Phytochemical analysis demonstrated the presence of alkaloids, tannin, terpenoidal saponins, steroidal saponins and glycosides in the active fractions. Odeyemi, (2014), has reported that *T. diversifolia* leaf, flower and root extracts have antibacterial activity due to the presence of metabolic compounds such as flavonoids, steroidal saponins and alkaloids. The observed anti-salmonella activity in the present study could be attributed by the presence of phytochemical compounds in the fractions investigated.

The MIC values of the four fractions E_A, E_B, E_C and E_D of ethyl acetate of *W. ugandensis* stem bark ranged from 1.22 to 312.5 µg/ml. In the present study, fraction E_C and E_D showed high anti-salmonella activity of 1.22 µg/ml against *S. ser. Typhimurium* (ATCC 1408), *S.* ser. Typhi (ATCC 13347) and *S. enterica* (ATCC 2162). In addition, fraction E_C and E_D had MIC values of 19.53 and 9.77 µg/ml respectively against *S. ser. Typhi* (ATCC 43579). Similarly, fraction E_A and E_B also exhibited appreciable amount of anti-salmonella activity. Fractions of ethyl acetate (stem bark) showed anti-salmonella activity against all clinical *Salmonella* isolates tested. Anti-salmonella activity of *W. ugandensis* fractions compared well with standard drug, ciprofloxacin broad spectrum antibiotics and there was no significant difference in the observed activities (P<0.0001). The observed anti-salmonella activity of *W. ugandensis* is supported by Yibeltal *et al*., (2013) who demonstrated activity of crude and semi-purified fractions of *W. ugandensis* against *Shigella boydii* and *Staphylococcous aureus*. Studies carried out by Olila *et al*., (2001), on aqueous extracts of *W. ugandensis* stem bark showed activity against both *Escherischia coli* and *Staphylococcous aureus* in agar well assays but not in disc diffusion assay. The anti-salmonella activity of *W. ugandensis* observed in the present study could be attributed to several secondary metabolites, among them steroids, terpenoids and glycosides.

The MIC values of seven fractions of hexane extracts of *W. ugandensis* root were determined. Fractions; H_A, H_B, H_C, H_D, H_E, H_F and H_G exhibited remarkable anti- salmonella activity in the range of 1.22 to 312.5µgml^{-1} against the 4 clinical

Salmonella isolates which is quiet comparable to ethyl acetate fractions. Fraction H_D showed higher anti-salmonella activity against three of the four clinical *Salmonella* isolates. Fraction H_D had MIC value of 1.22 µg/ml against *S. ser. Typhi* (ATCC 13347), *S. ser. Typhi* (ATCC 43579) and *S. enterica* (ATCC 2162). It also exhibited MIC value of 39.06µg/ml against *S. ser. Typhimurium* ATCC 1408. Fraction H_E demonstrated the least anti-salmonella activity against three out of the four isolates tested. This fraction had MIC values of 312.5, 156.25 and 312.5 µgml^{-1}against *S. ser. Typhi* (ATCC 13347), *S. ser. Typhi* (ATCC 43579) and *S. enterica* (ATCC 2162) respectively. However, it showed MIC value of 19.53 µg/ml against *S. ser. Typhimurium* (ATCC 1408). *Warbugia ugandensis* root fractions had the highest anti-salmonella activity. Anti-salmonella activity of *W. ugandensis* (root) fractions compared well with the activity of ciprofloxacin (1.22-19.53 µg/ml). The present study has demonstrated that MIC values for *W. ugandensis* fractions (root) against *Salmonella* isolates are comparable to those of ciprofloxacin. There was no significant difference in anti-salmonella activity for the fractions and that of ciprofloxacin ($p<0.0001$).The 1.22-19.53 µg/ml values exhibited by H_A-H_G have comparable activity with ethyl acetate fractions.

The present study has demonstrated lower MIC values for *W. ugandensis* hexane fractions (root) against *Salmonella* isolates tested than what Yibeltal *et al*., (2013), reported. According to their report, MIC values of semi-purified fraction of petroleum ether extract of *W. ugandensis* (heartwood) against both *S. boydii* and *S. aureus* was 500 µg/ml (0.5mg/ml) and 1000 µg/ml (1mg/ml) against *E.coli*. In addition, *Candida albicans* growth was inhibited by 1000 µgml^{-1} of semi-purified fraction of petroleum ether extracts of *W. ugandensis* both the leaves and the heartwood (Yibeltal *et al*., 2013). Results of growth inhibitory activity exhibited on the clinical isolates of *Salmonella* isolates by hexane fractions of *W. ugandensis* root indicated the plant contained anti-salmonella agents which supported its use in the local treatment of typhoid fever.

The result of phytochemical screening of the active chromatographic fractions for *W ugandensis* root revealed presence of alkaloids, steroidal saponins, terpenoidal saponins and glycosides. The flavonoids and tannins were absent in the fractions

analyzed. Ogoti *et al*., (2015), in their study, reported the presence of steroidal and terpenoidal in the hexane extracts of *W. ugandensis* root, which is similar to present study. The observed anti-salmonella activity of fractions of *W. ugandensis* roots in the present study could be attributed to several phytochemical compounds, among them steroidal, terpenoidal saponins and glycosides. These phytochemical compounds have been demonstrated to have anti-salmonella activity (Odeyemi, 2014; Ogoti *et al*., 2015).

5.5 Effects of plant fractions on *S. ser. Typhimurium* Dihydrofolate reductase

Dihydrofolate reductase (DHFR) regenerates folic acid into its reduced form tetrahydrofolate which is fundamental to growth and multiplication of cells. According to Nicola *et al*., (2007), folate and its derivatives are important co-factors in synthesis of nucleotides for all organism. Synthesizing nucleotides require tetrahydrofolate (THF). THF is formed from Dehydrafolate (DHF) by action of DHFR. Thus, any drug that can selectively inhibit the bacteria DHFR inhibits and kill the bacteria. Trimethoprim [2, 4-diamino-5-(3, 4, 5-trimethoxybenzyl) pyrimidine] is DHFR inhibitor mainly used as a folic acid antagonist and anti-infecting agent (Hawser *et al*., 2006). Its selective bactericidal activity involves blocking folate synthesis in the bacterial thymidine synthesis pathway, therefore disrupting DNA replication process. It is administered orally and 10-20% is metabolized in the liver. Trimethoprim (TMP) is active against most strains of aerobic gram positive and negative microorganisms, such as *Staphylococcus*, *Entobacter* species, *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*. Therefore, it is prescribed for treating urinary, intestinal and respiratory tract infections.

In this study, an attempt was made to determine the effect of plant fractions with known anti-salmonella properties on DHFR enzyme. Inhibition effect of DHFR was carried out using extracted DHFR from *S. ser. Typhimurium* (ATCC 1408) and was followed spectrophotometrically conversion of NADPH to $NADP^+$. Methanol fractions namely T_A, T_B, T_C, T_D, T_F and T_G, of *T. diversifolia* leaf were investigated for their effects on DHFR activity *in vitro*. All fractions tested were inhibitory to DHFR, resulting to more than 83% inhibition. Fractions T_A, T_B, T_C, T_F

(all doses), T_D (512mg/kg), and T_G (512, 256mg/kg) inhibited the enzyme completely. Both fractions T_D (128 and 256mg/ml) and T_G (128 mg/kg) showed the same specific activity (0.2338 ±0.02 µmol/min/mg) and this demonstrated an inhibitory effect of 83.3%. Trimethoprim, positive control had inhibition effect on the DHFR extracts of 96.1% (0.0779±0.12 µmol/min/mg) that was comparable to methanol fractions of *T. diversifolia* leaf. Fractions evaluated demonstrated inhibitory effect on the DHFR.

Hexane fractions namely H_A, H_B, H_C, H_D, H_E, H_F and H_G of *W. ugandensis* root exhibited inhibitory effects on DHFR activity. It was demonstrated that some hexane fractions of *W. ugandensis* root had appreciable amount of activity in an *In vitro* study on inhibition of DHFR varying from 0.1169±0.58 to 1.169±0.3 µmol/min/mg protein (Inhibitory effect of 94.2% and 41.7% respectively). All the fractions screened had inhibition effect on DHFR activity that was observed to be more than 41.7% (1.169±0.58 µmol/min/mg). Fractions H_A (256, 512mg/ml), H_B (512mg/ml), H_E (128,256, 512mg/ml), H_F (512mg/ml) and H_G (128,256, 512mg/ml) had complete (100%) inhibitory effect on DHFR activity. Trimethoprim had activity of 0.0779±0.12 µmol/min/mg (96.1% inhibition effect) while untreated activity of the DHFR was 2.004 µmol/min/mg (0% inhibitory effect). Hexane fractions of *W. ugandensis* root exhibited mixed inhibitory effects. Hexane column chromatograpy fractions have been shown to possess anti-salmonella activity with inhibition on DHFR. Fraction H_B (128mg/ml) had decreased activity to 0.1169±0.58 µmol/min/mg protein (94.2% inhibitory effect) and most of the fractions reduced to 0.2338±0.02 µmol/min/mg protein (88.3% inhibitory effect). Fraction H_F (128mg/ml), decreased activity to 1.169±0.3 µmol/min/mg protein (41.7 % inhibitory effect).

Ethyl acetate fractions of *W. ugandensis* stem bark namely E_A, E_B, E_C and E_D exhibited inhibitory effect on DHFR activity. The inhibitory effects of the 4 fractions screened were in the range of 82.5-100% at three doses tested. With fractions E_A (512mg/ml), E_C (512, 256mg/ml) and E_D (512,256, 128 mg/ml) DHFR activity was not detected. The fractions showed complete inhibitory effect on DHFR activity (100% inhibition). Likewise, trimethoprim, used as a positive control is a known inhibitor for DHFR had inhibitory effect of 96.1 % (0.0779±0.12 µmol/min/mg) and

this demonstrated that some enzymatic activity of the DHFR was taking place. The activity of DHFR enzyme subjected to fraction E_B at three different doses had inhibitory effect of 88.3% (0.2338±0.02 µmol/min/mg) to 82.5% (0.3508± µmol/min/mg) respectively. Meanwhile, the activity of DHFR enzyme without plant fractions or trimethoprim were determined as 2.004 µmol/min/mg (0% inhibitory effect). It was demonstrated that the 4 fractions had mixed inhibitory effect on DHFR enzyme at three different doses.

Methanol column chromatography fractions of *T. diversifolia* leaf, Hexane column chromatography fractions and ethyl acetate fractions of *W. ugandensis* root and stem bark respectively have been demonstrated to have inhibitoty effect on DHFR and are thought to block the enzyme causing cell death as result of DNA synthesis inhibition. For this reason, DHFR is considered an excellent target for anti-salmonella drugs. Likewise, trimethoprim is thought to bind more tightly to bacterial DHFR (Feeney *et al*., 2011).

Evidence from the present study clearly show that fractions of *T. diversifolia* leaf and *W. ugandensis* root and stem bark inhibit DHFR activity. Studies on inhibitoty effect of methanol plant extracts on DHFR have been done by Ahmad *et al*., (2011). Ahmad reported that the methanol extracts of *T. chebula* and *Q. infectoria* completely (100%) inhibited DHFR activity. Moreover, Juma *et al*., (2015), demonstrated the effect of sesquiterpene lactones from *Ocimum basilicum L* on DHFR *Plasmodium falciparum* and reported that sesquiterpene lactones in inhibit DHFR. This evidence support the present study on inhibitory effect of plant fractions on DHFR activity. As mentioned earlier, the inhibitors of DHFR are potential drugs for treatment of cancer, malaria, leishmanial, trypanosomiasis as well as bacterial infection (Navarro-Peran *et al*., 2005). Thus, the present study provides scientific support for the use of *T. diversifolia* and *W. ugandensis* as the source of anti-salmonella plants. Moreover, the fact that fractions of these plants were both active in the anti-salmonella and inhibitory effect on DHFR, suggests that the mechanism of action of the plant fractions is through DHFR inhibition. The selectivity of inhibitors is based on the fact that the bacteria cannot use pre-formed folic acid and must synthesis their folic acid *de novo*. In contrast, mammalian cells use folic acid obtained from food (Mayer, 2010). For instance, Sulfonamides and Sulfones are

antimicrobials that are analogue of para-aminobenzoic acid and competitively inhibit pteridine synthetase and therefore block the formation of dihydropteroic acid. They have broad range of activity against gram-positive and gram-negative bacteria. Likewise, trimethoprim, methotrexate and pyrimethamine bind to dihydrofolate reductase and inhibit formation of tetrahydrofolic acid. In biological systems, DHFR enzyme catalyzes the formation of tetrahydrofolate by reduction of dihydrofolate using NADPH as a cofactor. Tetrahydrofolate and its one carbon adducts are required for *de novo* synthesis of purines and thymidylate, as well as some amino acids. DHFR inhibition causes disruption of purine and thymidylate biosynthesis and DNA replication, leading to cell death (Abali *et al*., 2008). The antibiotics have broad range of activity against gram-positive and gram-negative bacteria and are used in combination with sulfonamides; this combination blocks two distinct steps in folic acid metabolism and prevents the emergence of resistant strains (Gallo *et al*., 2013). Therefore future isolation and characterization of these fractions of *T. diversifolia* leaf and *W. ugandensis* stem bark and root, new leading anti-salmonella compounds could be found.

5.6 GC-MS analysis of plant fractions

A total of eighteen semi purified fractions from methanol extracts of *T. diversifolia* leaf, ethyl acetate stem bark and hexane root extracts of *W. ugandensis* were analyzed by GC-MS technique to identify the compounds in column chromatography fractions. The study showed the presence of alkenes, fatty acids and short chain unsaturated carboxylic acid in the anti-*Salmonella* active methanol, ethyl acetate and hexane fractions. Nine important compounds were identified in methanol fractions based on the database in the NIST library. For instance, 9, 12-octadecadienoic acid (Z, Z) and n-hexadecanoic acid identified by GC-MS analysis are fatty acids whereas 3, 4, 5-trimethyl-1-hexene detected was alkenes. Crotonic acid was identified in fraction TE is a short chain unsaturated carboxylic acid, among others. Meanwhile, eight important compounds were detected in ethyl acetate stem bark extract of *W. ugandensis*. Some of them include; hexanedioic acid, bis-(2-ethylhexyl) ester, Hexadecanoic acid and E-15-heptadecanal. Likewise sixteen important compounds were also successfully

identified in the hexane extract of *W. ugandensis* root. E-15-heptadecenal, 1, 2-benzenedicarboxylic acid and 6-ethyl-2-methyldecane were detected among others.

Majority of the phytoconstituents identified in methanol, ethyl acetate and hexane extracts are attributed with various biological activities. For example, hexadecanoic acid is a very common saturated fatty acid, known anti-inflammtory phytoconstituent as it is a phospholipase inhibitor (Aparna *et al*., 2012). It's also known for its antibacterial activity (Manilal *et al*., 2009). The n-hexadecanoic acid is also a known fatty acid that possesses antioxidant, hypocholesterolemic, nematicide, pesticide and antiandrogenic activity (Duke, 2007). Likewise, 9,12-octadecadienoic acid (Z,Z), is otherwise called as omega 6 fatty acids which are a family of pro-inflammatory, anti-inflammatory polyunsaturated fatty acid and antimicrobial agents (Marimuthu *et al*., 2014). The 1, 2-benzenedicarboxylic acid and beta-sesquiphellandrene have been shown to have antimicrobial property (Duke *et al.*, 2007; Vukovic *et al*., 2007). E-15- Heptadecenal, an aldehyde was identified in both hexane and ethyl acetate extract of *W. ugandensis*, has been reported for antibacterial activity (Vinay *et al*., 2011).

Plant *Warburgia ugandensis* possess antiplasmodial properties. It also contains chiefly sesquiterpenes which contributed towards the antimicrobial profile of plant. Were *et al*. (2015), conducted study through Fourier Transform Infra-red Spectrometry to confirm presence of other compounds including alkaloids, terpenoids and terpenes in *W. ugandensis*. Study revealed the presence of N-H, C-H, C=C and C=O which proved presence of groups like; carboxylic acids, amines and alkenes. These functional groups established presence of compounds such as; alkaloids, terpenes and flavonoids. The study also concluded these constituents as responsible for the antiplasmodial activity (Were *et al*., 2015).

Tithonia diversifolia (Hemsl.) A. Gray, or Mexican sunflower, Asteraceae, is a prolific shrub that is known for its medicinal importance. Scientific research has established that skincare products formulated from *Tithonia diversifolia* extracts had remarkable antimicrobial properties (Kareru *et al*., 2010). It has been reported that dichloromethane leaf and flower extracts of *T. diversifolia* have antiplasmodial (Muganga *et al*., 2010). In general, the genus *Tithonia* is an important source of

diverse natural products, particularly sesquiterpene lactones, diterpenes, and flavonoids. They are known to possess wide variety of biological and pharmacological activities such as antimicrobial, cytotoxic, anti-inflammatory, antiviral, antibacterial, antifungal activities, effects on the central nervous and cardiovascular systems as well as allergenic potency (Chagas-Paula *et al*., 2012). The non-volatile fractions of the plant are a rich source of flavonoids and sesquiterpenoid lactones, including 8-(2-methylbutanoyl)-3, 10-epoxy-3, 8-dihydroxyl-4, 11 (13)-germacradien-12, 6-olide, tagitinin A, tagitinin C, tagitinin F, acetyl tagitinin E, diversifolol, diversifolin, tirotundin and hispidulin (Gu *et al*., 2002).

The present study shows the presence of alkenes, fatty acids, short chain unsaturated carboxylic acid, flavonoids and terpenoidal saponins in the active methanol, ethyl acetate and hexane fractions of the plants investigated. The phytochemical analysis of *T. diversifolia* leaf and *Warburgia ugandensis* extracts revealed the presence of alkoloids, tannins, flavanoids, steroidal saponins, terpenoidal saponins and glycosides. The presence of compounds in alkaloidal and terpenoidal saponins families were confirmed by GC-MS analysis.

5.7 Conclusion

- The present study demonstrated that the methanol extract of *T. diversifolia* leaf, ethyl acetate and hexane extracts of stem bark and root of *W. ugandensis* respectively contains phytochemical compounds such as alkaloids, tannins, flavonoids, steroidal saponins, terpenoidal saponins and glycosides.
- The *in vitro* evatuation of methanol *T. diversifolia* leaf, ethyl acetate and hexane of stem bark and root of *W. ugandensis* extracts respectively showed potent anti-salmonella activity.
- Extracts from *T. diversifolia* and *W. ugandensis* namely TDLE, TDFH, TDLM, TDLH, TDFE, WURE, WURH, WUSBE and WUSBH evaluated for acute and sub-chronic toxicity in Swiss white mice did not exhibit any significant toxicity.
- The nine extracts that demonstrated *in vitro anti*-salmonella activity were also tested for *in vivo* anti-salmonella activity in mice, experimentally infected with *S. ser. Typhymurium* (ATCC 1408). *In vivo* studies showed that only three extracts; methanol extract of *T. diversifolia* leaf, ethyl acetate and hexane extracts of stem bark and root respectively of *W. ugandensis* were observed to have anti-salmonella activity in mice.
- Minimum Inhibitory Concentrations (MIC) values of the s column chromatography fractions of methanol, ethyl acetate and hexane were found to be in range of 1.22to 312.5µg/ml. These results were comparable with that of ciprofloxacin (1.22-19.53µg/ml). Evidence from the present study clearly show that fractions of the two plants inhibit DHFR activity and demonstrated high potency as anti-salmonella agent.
- Thirty three known compounds were identified from the methanol, ethyl acetate and hexane fractions by GC-MS and grouped as alkenes, fatty acids, short chain unsaturated carboxylic acid, flavonoids and terpenoidal saponins. Among them n-hexadecanoic acid, 9, 12-octadecadienoic acid, 1, 4, 8-cycloudecatriene-2, 6, 6-carophylene, ß-sequiphellandrene and 3-ethenyl-3-methyl-2, 1-(1-methylethenyl) cyclohexanol were found to have anti-salmonella activity.

5.8 Recommendations

It is therefore recommended that:

- The fractions be further purified using the activity guided isolation, characterization and structure elucidation of the pure compound(s) from methanol *T. diversifolia* leaf, ethyl acetate stem bark and hexane root of *W. ugandensis*.
- *In vitro* and *in vivo* investigations of purified compound(s) to be conducted against drug resistant clinical *Salmonella* isolates.
- The pure compound(s) be tested for inhibitory effect against *S. ser. Typhimurium* dihydrofolate reductase.
- Kinetic studies of purified compound(s) against *S. ser. Typhimurium* dihydrofolate reductase to be carried out to enhance understanding of mechanisms of action.

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APPENDICES

Appendix i: Scientific classification of plants

a) *Tithonia diversifolia*

Kingdom:	Plantae
(unranked):	Angiosperms
(unranked):	Eudicots
(unranked):	Asterids
Order:	Asterales
Family:	Asteraceae
Genus:	*Tithonia*
	T. diversifolia
Species:	

b) *Wurbugia ugandensis*

Kingdom:	Plantae
(unranked):	Angiosperms
(unranked):	Magnoliids
Order:	Canellales
Family:	Canellaceae
Genus:	*Warburgia*
Species:	*W. ugandensis*

c) *Croton megalocarpus*

Kingdom:	Plantae
(unranked):	Angiosperms
(unranked):	Eudicots
(unranked):	Rosids
Order:	Malpighiales
Family:	Euphorbiaceae
Subfamily:	Crotonoideae
Tribe:	Crotoneae
Genus:	*Croton*
Species	*Croton megalocarpus*

d) *Carissa edulis*

Kingdom	Plantae – Plants
Subkingdom	Tracheobionta – Vascular plants
Superdivision	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Subclass	Asteridae
Order	Gentianales
Family	Apocynaceae – Dogbane family
Genus	*Carissa* L. – carissa P
Species	*Carissa edulis*

e) ***Launaeacornuta***

Kingdom	Plantae – Plants
Subkingdom	Tracheobionta – Vascular plants
Superdivision	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Subclass	Asteridae
Order	Asterales
Family	Asteraceae – Aster family
Genus	*Launaea* Cass. – launaea P
Species	*Launaea cornuta*

Appendix ii: Letter of approval of ethic clearance

JOMO KENYATTA UNIVERSITY
OF
AGRICULTURE AND TECHNOLOGY

RESEARCH, PRODUCTION AND EXTENSION DIVISION
P. O. Box 62000-00200 Nairobi-Kenya Tel: (067)–52711, 52181/4
Fax: 067 – 52030, Email: research@rpe.jkuat.ac.ke

From: Chairman, Biosafety and Research Ethics Committee

Date: 10th August 2011

To: Mr Peter Ogoti Mose
CPC

Ref: JKU/2/49/006

RE: ETHICAL CLEARANCE TO CONDUCT A PH.D RESEARCH WORK ENTITLED ' BIO-PROSPECTING FOR EFFECTIVE ANTIBIOTICS AGAINST *SALMONELLA TYPHIMURIUM* FROM SELECTED KENYAN MEDICINAL PLANTS'

This is to inform you that the University Biosafety and Research Ethics Committee (BREC) has received your request dated 2nd August 2011, seeking ethical clearance to conduct a PhD. Project entitled: '**Bio-prospecting for effective antibiotics against *Salmonella typhimurium* from selected Kenyan medicinal plants**'. The study will be carried out in JKUAT Biochemistry department. The research work involves the use of experimental animals for the purpose of studying the toxicity and efficacy levels of the plant extract. The committee considered the ethical and biosafety issues with respect to the experimental setups, material handling and any human and environmental interactions.

The approval is hereby **granted** to you to proceed on with research work from the date of this letter. Please take note that any changes to the research study must be reported to this committee prior to implementation. This includes changes to research design, equipments, personnel, funding or procedures that could introduce new or more than minimum risk to research participant. In the course of your study you will be required to fill in ethical compliance form to be issued from RPE.

We wish you success in this study.

Yours sincerely,

Dr. Julius Maina Mathara (Ph.D)
Chairman, Biosafety and Research Ethics Committee, JKUAT

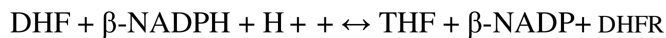
Copy to: Principal Research officer JKUAT
Director BPS, JKUAT
Chairman, Department of Biochemistry, JKUAT

JKUAT is ISO 9001: 2008 Certified. | **Setting Trends in Higher Education, Research and Innovation**

Appendix iii: Summary of enzymatic assay of *S. ser. Typhimurium* DHFR

a. Principle

Dihydrofolate reductase (DHFR) is a ubiquitous enzyme present in all eukaryotic and prokaryotic cells, playing a key role in thymidine synthesis. It catalyzes the reduction of 7,8dihydrofolate (DHF) to 5,6,7,8 tetrahydrofolate (THF), utilizing NADPH as cofactor. This reaction is an essential step in the biosynthesis of nucleotidic bases of DNA. Blockage of the DHFR enzyme causes cell death as a result of DNA synthesis inhibition. For this reason, DHFR is considered an excellent target for antitumor drugs.



DHF + β-NADPH + H + + ↔ THF + β-NADP+ DHFR

b. Reagents used

Crude Dihydrofolate Reductase, 50mM Potassium phosphate buffer, 2.3 mM Dihydrofolic acid, DHFR substrate (Product code A6770) and 0.11mM Nicotinamide Adenine dinucleotide phosphate (NADPH) reduced tetrasodium salt) and 0.1%(w/v) Bovine Serum Albumin Solution(BSA).

i) Preparation of reagents

50mM Potassium phosphate buffer

- 100ml of potassium phosphate (monobasic) solution was prepared using deionized water and the pH was adjusted at 25°C with 1 M KOH.

0.11mM β-NADPH reduced tetrasodium salt

- Dissolved the contents of one 5 mg vial of β-NADPH reduced form, Tetrasodium salt in appropriate volume of 50 mM Potassium phosphate buffer solution. This was freshly prepared before use.

2.3 mM Dihydrofolic acid

- 1 ml of Potassium phosphate buffer solution was immediately prepared using dihydrofolic acid and dissolution was facilitate with 1 M KOH(0.020 ml of 1 M KOH per mg of dihydrofolic acid.This solution only stable for 10 minutes and it was freshly prepared before use.

0.1 %(w/v) Bovine Serum Albumin Solution (BSA)

- Prepared 100ml in potassium phosphate buffer using albumin bovine.

Dihydrofolate reductase enzyme extract preparation

- Immediately before use DHFR enzyme extract solution was prepared in cold Bovine Serum Albumin Solution.

ii) **Conditions:**

T = 25°C, pH = 6.5, A340nm, Light Path = 1 cm

iii) **Method:**

Continuous Spectrophotometric Rate Determination

iv) Final assay concentration

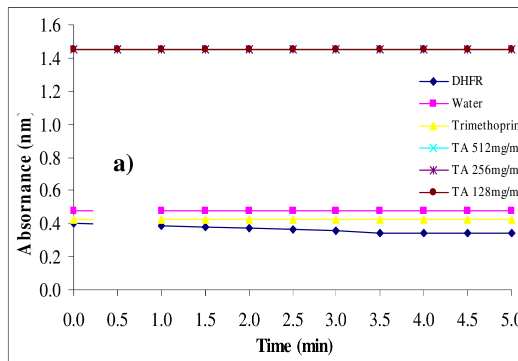
In a 3.20 ml reaction mix, the final concentrations are 50 mM potassium phosphate, 0.072 mM dihydrofolic acid, 0.10 mM β-NADPH reduced form, 0.003% (w/v) bovine serum albumin and 2.2 mg/ml dihydrofolate reductase.

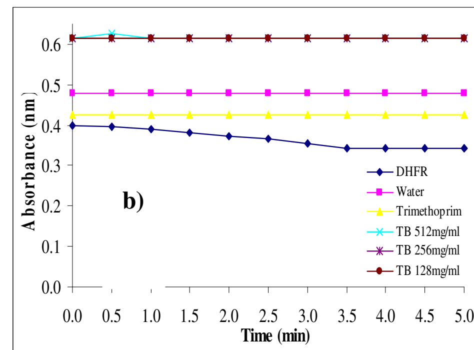
Appendix iv: Rate of reactions of plant fractions on DHFR

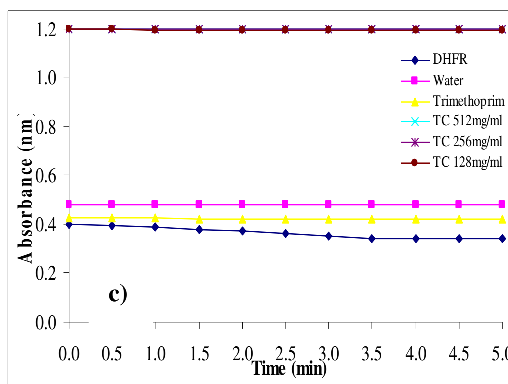
a) Reaction curves of plant fractions on DHFR

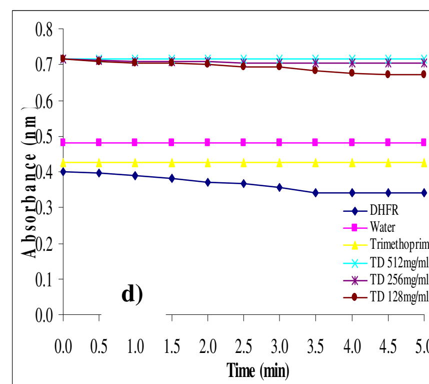
i) Methanol fractions of *T. diversifoilia* leaf

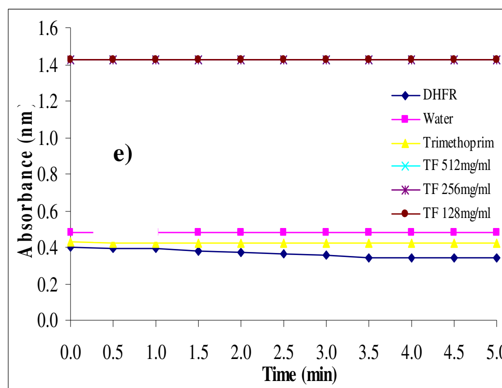
The curves below depict the results obtained on inhibition of *S.*ser. Typhimurium DHFR activity by T_A, T_B, T_C, T_D, T_F, and T_G of methanol fractions of *T. diversifolia* leaf respectively. The fractions had varied rate of reactions for the three different concentrations evaluated within 5 minutes period. Rate of reaction for DHFR (0.1ml) exhibited a linear slope during the 3.5 minutes of detection and absorbance at 340nm indicated decrease (due to decrease in NADPH). Inhibition for active fractions occurred within seconds which compared well with trimethoprim (positive control).

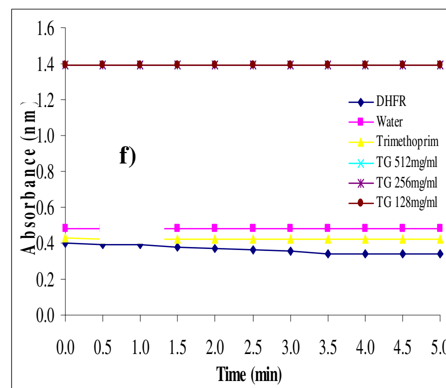








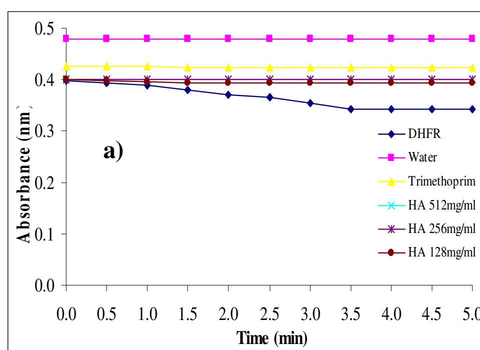


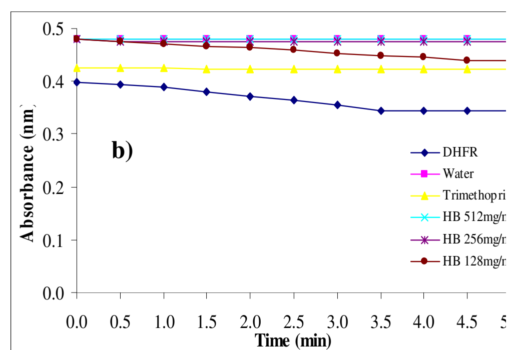


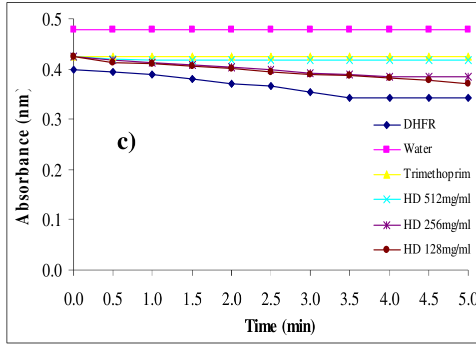
Key: Rate of reaction of (a) T_A (b) T_B (c) T_C (d) T_D (e) T_F and (g) T_G on DHFR actvity

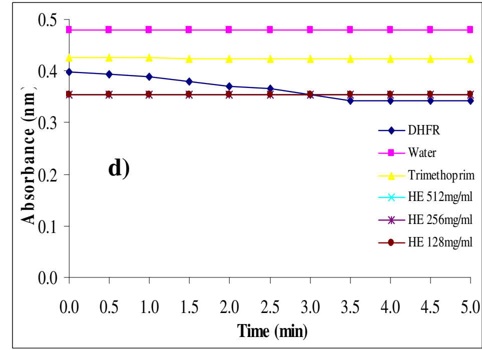
ii) Haxane fractions of *W. ugandensis* root

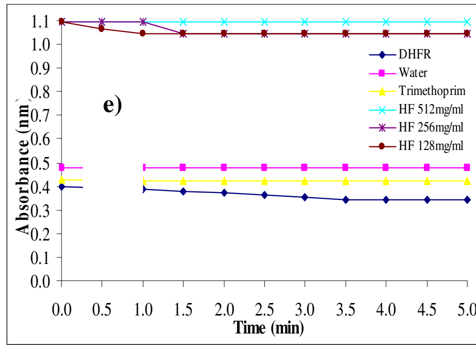
Curves; (a), (b), (c), (d), (e), (f), (g) and (h) rate of reaction of 8 hexane fractions of *W. ugandensis* root (H_A, H_B,H_D, H_E,H_F,H_G, H_H and H_I respectively)against DHFR enzyme. The results obtained for the fractions demonstrated varied rate of reactions due to different degree of inhibition, perhaps, fractions contained different active compounds. The activity of DHFR was observed within 3.5 minutes of the reaction. Likewise the inhibitory effect of trimethoprim was detected within seconds. However, fraction H_F (256mg/ml) indicated inhibitory effect within zero to one minute and certainly a reaction took place after 1 minute for ½ minute while at 128mg/ml there was immediate reaction for 1½ minutes.

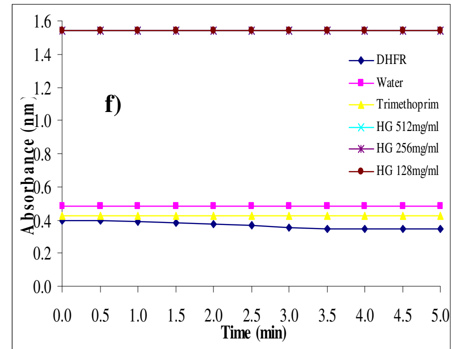


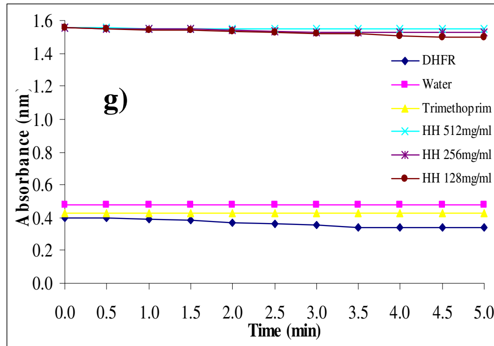


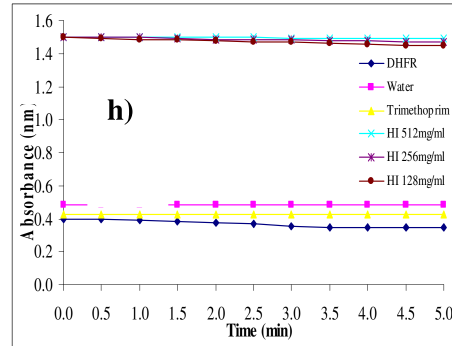

c)
Absorbance (nm)
Time (min)
DHFR
Water
Trimethoprim
HD 512mg/ml
HD 256mg/ml
HD 128mg/ml


d)
Absorbance (nm)
Time (min)
DHFR
Water
Trimethoprim
HE 512mg/ml
HE 256mg/ml
HE 128mg/ml


e)
Absorbance (nm)
Time (min)
DHFR
Water
Trimethoprim
HF 512mg/ml
HF 256mg/ml
HF 128mg/ml

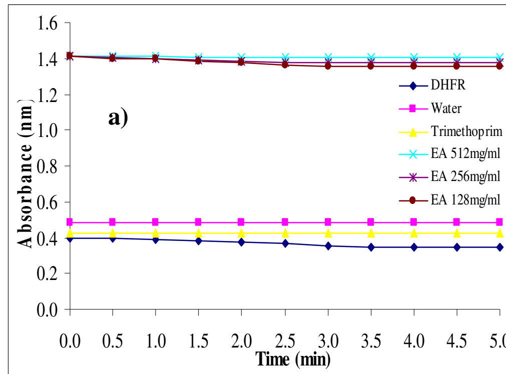

f)
Absorbance (nm)
Time (min)
DHFR
Water
Trimethoprim
HG 512mg/ml
HG 256mg/ml
HG 128mg/ml

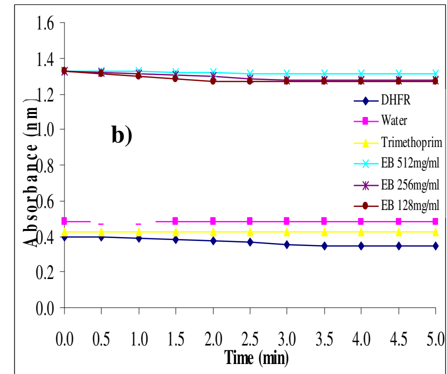

g)
Absorbance (nm)
Time (min)
DHFR
Water
Trimethoprim
HH 512mg/ml
HH 256mg/ml
HH 128mg/ml

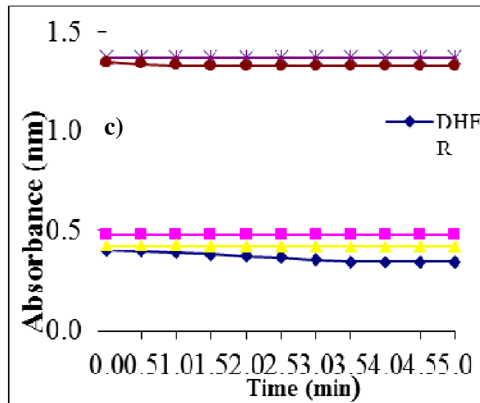

h)
Absorbance (nm)
Time (min)
DHFR
Water
Trimethoprim
HI 512mg/ml
HI 256mg/ml
HI 128mg/ml

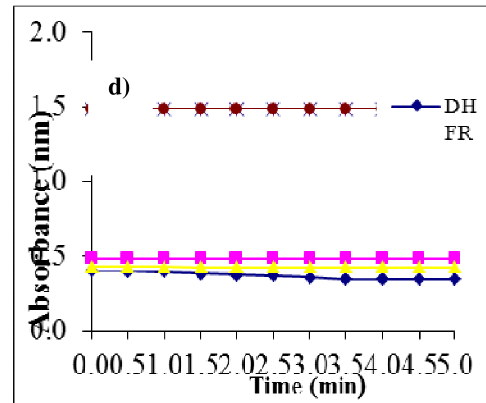
iii) Ethyl acetate fractions of *W. ugandensis* stem bark

Curves, (a), (b), (c), and (d) show the inhibitory effect of 4 ethyl acetate fractions of *W. ugandensis* stem bark against *S. ser. Typhimurium* DHFR enzyme. The four fractions exhibit diferent rate of reactions on DHFR enzyme.









b) Summary of rate of reaction (nm/min) of plant fractions and controls

Sample	Rate of reaction(nm/min) of different extract concentrations		
	128mg/ml	256mg/ml	512mg/ml
DHFR	1.60×10^{-2a}	1.60×10^{-2a}	1.60×10^{-2a}
Water	0.00^{c}	0.00^{c}	0.00^{c}
TMP	1.33×10^{-3b}	1.33×10^{-3b}	1.33×10^{-3b}
T_A	3.00×10^{-3b}	0.00^{c}	0.00^{c}
T_B	0.00^{c}	0.00^{c}	0.00^{c}
T_C	3.33×10^{-3b}	0.00^{c}	0.00^{c}
T_F	0.00^{c}	0.00^{c}	0.00^{c}
T_G	1.00×10^{-3b}	0.00^{c}	0.00^{c}
H_A	3.17×10^{-3b}	0.00^{c}	0.00^{c}
H_B	1.20×10^{-3a}	8.89×10^{-3a}	0.00^{c}
H_D	1.08×10^{-2a}	9.11×10^{-3a}	5.33×10^{-3b}
H_E	0.00^{c}	0.00^{c}	0.00^{c}
H_F	1.60×10^{-2a}	1.60×10^{-2a}	0.00^{c}
H_G	0.00^{c}	0.00^{c}	0.00^{c}
H_H	1.18×10^{-2a}	8.29×10^{-3a}	3.00×10^{-3b}
H_I	1.02×10^{-2a}	7.25×10^{-3a}	2.40×10^{-3b}
E_A	1.60×10^{-2a}	1.60×10^{-2a}	1.60×10^{-2a}
E_B	1.60×10^{-2a}	1.60×10^{-2a}	1.60×10^{-2a}
E_C	1.33×10^{-2a}	0.00^{c}	0.00^{c}
ED	0.00^{c}	0.00^{c}	0.00^{c}
TD	9.56×10^{-3a}	3.14×10^{-3b}	0.00^{c}

Mean values of the rate of reaction of the samples. The means with different superscript letters are significantly different, $p>0.0001$

Appendix v: Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) analyse of the methanol extracts of *T. diversifolia* leaf, ethyl acetate extracts of *W. ugandensis* stem bark and hexane extracts of *W. ugandensis* root were done using pre-coated silica gel (Merck, S 0,032-0,063mm). A combination of Dichloromethane, Chloroform and Ethyl acetate in the ratio of 3:2:1 was used as solvent mixture. The pre-coated silica aluminium plates were used for TLC studies of secondary metabolites. The fractions of the extracts mentioned above were run and spotted on the plate. The plates were placed in TLC chamber and the chromatogram was developed with solvent mixture. The TLC plates were taken out and visualized with UV lamp fluorescent at 366nm and 254nm visible light. The spots were marked and the migration pattern was recorded.

Appendix vi: Gas chromatography-Mass spectrometry (GC-MS) analysis

Fragmentation patterns of selected eluted components of plant fractions

With an electron beam of 9 – 15 eV the principle ion produced is the molecular ion, which is produced by the loss of a single electron. This gives a very simple spectrum with essentially the entire ion appear with the parent peak. With organic compounds, because of the small but observable natural abundance of carbon 13 and tritium there is a small peak appearing one mass unit higher than the parent peak (M+1) and if two isotopes happen to be in the same molecule there is even a smaller peak at M+2. The base peak is the largest peak observed and all other peak heights are measured with respect to it. Thus, ion abundances are given in terms of ions produced and the relative strength of these ions is related to the strength and chemical nature of the bonds which held the fragments to the rest of the molecule. Cleavage is usually favored at branched carbon atoms as a consequence of the stability of the carbonion ions produced that is tertiary > secondary > primary. It is also favored by the formation of small stable molecule like water and CO.

Appendix vii: Statistical analysis of parameters

The following indicate summaries of data analysed by ANOVA and T-test

a) ANOVA summary

i) Activity of plant extracts administered orally against *S*. ser. Typhimurium in Swiss white mice

Source	DF	Sum of squres	Mean squares	F value	Pr>F	R-squares	Coeff Var
Model	28	10103.64	360.84	8.37	<0.0001	0.67	61.59

ii) Acute toxicity test analysis

Dependent variable: Body weights

Source	DF	Sum of squres	Mean squares	F value	Pr>F	R-squares	Coeff Var
Model	9	1199.65	133.29	39.55	<0.0001	0.51	0.04

Dependent variable: Packed Cell Volume

Source	DF	Sum of squres	Mean squares	F value	Pr>F	R-squares	Coeff Var
Model	9	1026.87	114.10	12.89	<0.0001	0.25	6.4

b) T-test

Two Sample T-test	t	df	Sig(2-tailed)...
TDLM 300mg/kg - TDLM 600mg/kg ...	5.333	54	0
TDLM 300mg/kg - TDLM 1200mg/kg ...	14.54	54	0
TDLM 600mg/kg - TDLM 1200mg/kg ...	6.222	54	0
TDLE300mg/kg - TDLE600mg/kg ...	3.174	54	0.002
TDLE300mg/kg - TDLE1200mg/kg ...	1.073	54	0.288
TDLE600mg/kg - TDLE1200mg/kg ...	-1.702	54	0.095
WURH 300mg/kg - WURH 600mg/kg ...	-0.923	54	0.36
WURH 300mg/kg - WURH 1200mg/kg ...	2.655	54	0.01
WURH 600mg/kg - WURH 1200mg/kg ...	1.405	54	0.166
WURE 300mg/kg - WURE600mg/kg Mice ...	-6.267	54	0
WURE 300mg/kg - WURE1200mg/kg ...	-4.244	54	0
WURE600mg/kg Mice - WURE1200mg/kg..	2.103	54	0.04
WUSBE300mg/kg - WUSBE600mg/kg ...	-0.144	54	0.886
WUSBE300mg/kg - WUSBE 1200mg/kg ...	0.102	54	0.919
WUSBE600mg/kg - WUSBE 1200mg/kg ...	0.291	54	0.772
WUSBH300mg/kg - WUSBH600mg/kg ...	-4.199	54	0
WUSBH300mg/kg - WUSBH1200mg/kg ...	-2.86	54	0.006
WUSBH600mg/kg - WUSBH1200mg/kg ...	3.738	54	0
TDLH300mg/kg - TDLH600mg/kg ...	-21.537	54	0
TDLH300mg/kg - TDLH1200mg/kg ...	-20.596	54	0
TDLH600mg/kg - TDLH1200mg/kg ...	1.165	54	0.249
TDFH300mg/kg - TDFH600mg/kg ...	2.426	54	0.019
TDFH300mg/kg - TDFH 1200mg/kg ...	4.341	54	0
TDFH600mg/kg - TDFH 1200mg/kg ...	3.117	54	0.003
TDFE 300mg/kg - TDFE 600mg/kg ...	0.178	54	0.86
TDFE 300mg/kg - TDFE1200mg/kg ...	-1.007	54	0.318
TDFE 600mg/kg - TDFE1200mg/kg ...	-0.985	54	0.329

Appendix viii: Publications

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In vitro anti-*Salmonella* activity of extracts from selected Kenyan medicinal plants

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The aim of this study was to determine *in vitro* anti-*Salmonella* activity of extracts of five selected Kenyan medicinal plants against *Salmonella* ser. Typhi and *Salmonella* ser. Typhimurium. The extracts from *Tithonia diversifolia*, *Warburgia ugandesis*, *Croton megalocarpus*, *Carissa edulis* and *Launae cornuta* plants traditionally used in treatment of typhoid fever were screened for anti-*Salmonella* activity using disc diffusion and microdilution techniques. The results from the present study have shown that out of thirty six extracts investigated, only nine extracts from *T. diversifolia* and *W. ugendensis* showed activity against *Salmonella* ser. Typhi and *Salmonella* ser. Typhimurium at 1000 mg/ml. The inhibition zone of ethyl acetate, hexane and methanol extracts of *T. diversifolia* leaves, ethyl acetate and hexane extracts of *T. diversifolia* flowers, ethyl acetate and hexane extracts of *W. ugandensis* stem barks, ethyl acetate and hexane extract of *W. ugandensis* roots ranged from 8 to 18.5 ± 0 mm. These results were comparable with those of ciprofloxacin (19.67 to 26 mm) and chloramphenicol (6.67 to 24.33 mm). The minimum inhibitory concentration (MIC) of the active extracts were in the range of 0.031 to 15.63 mg/ml which compared very well with ciprofloxacin (0.015 to 0.02) and chloramphenicol (0.022 to 0.03 mg/ml). Extracts with anti-*Salmonella* activity can be used to source antibiotic substances useful in the treatment of typhoid fever. The study provides the scientific basis for the traditional application against typhoid fever.

Key words: Anti-*Salmonella* activity, medicinal plant extracts, minimum inhibitory concentration, disc diffusion technique, microdilution technique, *Salmonella* strains, typhoid.

INTRODUCTION

Salmonella serotype Typhimurium (*S.* ser. Typhimurium), is a Gram-negative bacterial pathogen that infects humans and animals, causing significant morbidity and mortality worldwide (Fink and Cookson, 2007). It is an obligate intracellular bacterial pathogen that causes gastroenteritis in millions of people worldwide each year (Grassl et al., 2008). For instance, the Centre for Disease Control (CDC) estimates that there are nearly 1.4 million food-borne *Salmonella* infections annually in the USA (Mead et al., 1999). Various strategies have been

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employed in the treatment and management of *Salmonella* infection. Fluoroquinolones and tetracyclines are most commonly used to treat *Salmonella* infections. However, *Salmonella* strains resistant to these antibiotics have been reported in Korea and other countries (Choi et al., 2005; Stevenson et al., 2007). One major concern to public health has been the global dissemination of *S. typhimurium* Definitive Type 104, which is resistant to cotrimoxazole, nalidixic acid and ampicilin (Perron et al., 2008; Kariuki et al., 2010). The rise in antibiotic-resistant strains has led to increased interest in use of plant materials to develop new effective drugs. According to World Health Organization (WHO) more than 80% of world's population relies on traditional medicine for their primary healthcare, majority of who use plant active principles (Gupta et al., 2005). A wide variety of plants are used in Africa for treatment of fever, dysentry, cholera, diarrhoea and other infections typical of the tropical countries (Ayogu and Amadi, 2009; Ajayi and Akintola, 2010). For instance, traditional practitioners in Nigeria use herbal preparations to treat microbial infections such as typhoid and paratyphoid infections (Iroha et al., 2010).

Plants used in this study have traditionally been associated with disease curative and preventive practices in many countries for a long time. Garcia and Delgado (2006) have reported that *Tithonia diversifolia* has promising medicinal value. Skin products formulated from *T. diversifolia* extracts have been shown to have antimicrobial properties (Kareru et al., 2010).

In Ethiopia *Warburgia ugandensis* extracts are used to treat malaria, tuberculosis, bronchitis, pneumonia, hepatitis, tapeworm, gonorrhea and asthma (Wube et al., 2010; Were et al., 2010; Opiyo et al., 2011). The decoction from *Croton megalocarpus* bark is used as a remedy for worms and whooping cough. Grounded roots are used for syphilis, anthrax, and snakebites treatment (Kabir et al., 2005). Different communities in Africa use parts of *Carissa edulis* to alleviate pain, treat venereal diseases, glandular inflammation, induce abortion and restore virility (Githiori et al., 2004). In Kenya and Tanzania decoction from *Launae cornuta* roots is used as a remedy for cough, typhus fever and measles (Schippers, 2004).

This study investigated the anti-*Salmonella* activities of *T. diversifolia*, *W. ugandensis*, *C. megalocarpus*, *C. edulis* and *Launae cornuta*. Clinical isolates of *S.*ser.Typhi (ATCC 13347), *S.*ser.Typhi (ATCC 43579), *S.enterica* (ATCC 2162) and *S.* ser. Typhimurium (ATCC 1408) were used in the study.

MATERIALS AND METHODS

Salmonella strains

Clinical samples of *S.*ser.Typhi (ATCC 13347), *S.*ser.Typhi (ATCC 43579), *S.enterica* (ATCC 2162) and *S.* ser. Typhimurium (ATCC 1408) were provided by the Centre of Microbiology Research, Kenya Medical Research Institute (CMR-KEMRI) for this study.

Plant

The five plants selected for this study were collected in Nyamira County as indicated in Table 1. The plants were authenticated at Jomo Kenyatta University of Agriculture and Technology, Botany Department.

Experimental design

The nine plant parts obtained from 5 selected medicinal plants indicated in Table 1 were extracted using four solvent systems namely; hexane, ethyl acetate, methanol and water. The extracts obtained were subjected to standard phytochemical analyses as described by Jigna et al. (2006). In addition, the extracts were screened for anti-*Salmonella* activity against four clinical isolates. Samples in triplicate were subjected to disc diffusion and microdilition in duplicates to confirm anti-*Salmonella* activity.

Preparation of plant materials for extraction

The plant materials were washed under running tap water and left to drain off. The plant parts were chopped into small pieces. The dried pieces were ground into powder and their weights recorded.

Extraction techniques

The nine plant materials were extracted using selected solvents. Each plant material was extracted sequentially using hexane, ethyl acetate and methanol in the order of increasing polarity. Single extraction was carried out using water for each of the nine plant materials.

Preparation of hexane extract

Approximately 500 g of each plant powder was soaked separately in 1500 ml of hexane. The contents were kept for 3 days away from direct sunlight, undisturbed, then filtered through sterile filter paper. The filtrate was concentrated at 38.5 to 42°C.

Preparation of ethyl acetate extract

The hexane residues were re-soaked in 1500 ml of ethyl acetate. The contents were kept for 5 days away from direct sunlight, undisturbed and afterward filtered. The filtrate was concentrated at 38.5 to 42°C.

Preparation of methanol extract

The ethyl acetate residues were re-soaked in 1500 ml of methanol and kept for 36 h away from direct sunlight undisturbed. After filtration, the filtrate was concentrated at 65°C. The extracts were stored at 4°C until used.

Preparation of aqueous extract

Five hundred grams (500 g) of each of the powdered plant materials was weighed and soaked separately in 1500 ml of distilled water. The contents were warmed in a water bath for 2 h at

Table 1. Profile of the five medicinal plants.

Botanical name	Family name	Part of the plant used
Tithornia diversifolia	Solanaceae	Flowers and leaves
Warburgia ugandesis	Conellaceae	Roots and stem barks
Croton megalocarpus	Euphorbiaceae	Barks
Carissa edulis	Apocynaceae	Roots and barks
Launae cornuta	Asteraceae	Roots and leaves

60°C, then left to stand at room temperature for 10 h, undisturbed. They were subsequently sterile filtered and filtrate freeze dried to powder. The powder were weighed and stored until used.

Determination of phytochemical constituents

The freshly prepared extracts were subjected to standard phytochemical analyses for tannins, alkaloids, terpenoids, flavanoids, glycosides, steroids and saponin as described by Jigna et al. (2006).

Controls

Water and dimethyl sulfoxide (DMSO) were used as negative controls. Ciprofloxacin and chloramphenicol (Transchem pharmaceutical Ltd, Kenya) were used as positive controls.

Disc diffusion assay

Circular paper discs (6mm diameter) were placed on Muller Hinton media inoculated with *Salmonella* strains. Sterile paper discs were dampened with 10 µl of plant extracts at 1000 mg/ml. The loaded disc was placed on the surface of the medium, the compound was allowed to diffuse for 5 min and plates were incubated for 24 h at 37°C. Discs containing ciprofloxacin and chloramphenicol were used as positive controls. Discs loaded with DMSO and water served as negative controls. The assays were performed in triplicate. Anti-*Salmonella* activity was evaluated by measuring diameter of the inhibition zone.

Determination of minimum inhibitory concentration (MIC) values

The MIC values were determined using microdilution assay as described by Eloff (1998). Ciprofloxacin and chloramphenicol were used as positive controls and DMSO was used as negative control. Plant extracts were tested against *Salmonella* strains with varying concentration ranging from 62.5 to 0.0305 mg/ml. Briefly, 100 µl of sterile distilled water was added to each well of 96-well microtitre plates (SIGMA Aldrich, German) followed by the addition of 100 µl of 62.5 mg/ml and thereafter serially diluted plant extracts. Then 100 µl of *Salmonella* strains were added to each micro well to give a final volume of 200. The prepared plates were sealed to avoid drying and incubated overnight at 37°C. After overnight incubation, 50 µl of 5 mg/ml 2, 3, 5 triphenyltetrazolium chloride (SIGMA Aldrich, German) was added to the wells and incubated overnight. The pink colour was indicative of bacterial growth while lack of color was linked to growth inhibition. The MIC was defined as the lowest concentration of plant extract that completely suppress the growth of *Salmonella* strains.

Statistical analysis

Anti-*Salmonella* activity was determined from means of triplicates in zones of inhibition and duplicates in MICs. Collected data was analysed statistically using one way ANOVA (SAS, Version 9.0). Difference in values at $P < 0.0001$ were considered statistically significant.

RESULTS

Out of 36 plant extracts screened using disc diffusion assay, only nine extracts inhibited the growth of clinical Salmonella organisms at 1000 mg/ml. Extracts of hexane (flowers), ethyl acetate (leaves) and methanol (leaves) extracts from *T. diversifolia* were active against *S.*ser.Typhi ATCC 13347, *S.*ser.Typhi ATCC 43579, *S.enterica* ATCC 2162 and *S.*ser.Typhimurium ATCC 1408. Extracts of hexane (leaves) and ethyl acetate (flowers) from *T. diversifolia* inhibited growth of *S.*ser.Typhi ATCC13347. As was observed with ciprofloxacin and chloramphenicol controls, extracts of hexane and ethyl acetate (roots and stem bark) from *W. ugandensis* inhibited growth of all the tested *Salmonella* organisms. Extracts of methanol (leaves) from *T. diversifolia* were also observed to inhibit all the clinical isolates at 8 to 12 mm. The zones of inhibition for the active extracts are shown in Table 2.

The MIC values of the nine plant extracts was evaluated and shown to range from 0.031 to 15.63 mg/ml. The MICs of hexane extracts from *T. diversifolia* leaves and flowers ranged from 0.24 to 1.95 mg/ml and 0.12 to 3.91 mg/ml, respectively. The MICs of hexane extracts from *W. ugandensis* roots and stem bark ranged from 0.031 to 3.91 mg/ml and 0.031 to 0.488 mg/ml, respectively. Table 3 shows MICs of the nine plant extracts and controls. It is evident from these results that *W. ugandensis* extracts were the most active against all the *Salmonella* strains tested.

Table 4 illustrates anti-*Salmonella* activity values obtained from disc diffusion and microdilution methods. The nine plant extracts showed different value of anti-*Salmonella* activity against test strains. Ethyl acetate extract of *W. ugandensis* stem bark gave the lowest MIC value of 0.031 mg/ml against *S.*ser.Typhi ATCC 13347, *S.*ser.Typhi ATCC 43579, and *S.*ser.Typhimurium ATCC 1408 with zones of inhibition of 6, 7 and 7.33 mm,

Table 2. Zones of inhibition of clinical *Salmonella* strains by hexane, ethyl acetate and methanol extracts of selected medicinal plants

Plant extracts	Mean diameter of inhibition zones (mm)			
	S.ser.Typhi ATCC 13347	S.ser.Typhi ATCC 43579	S.enterica ATCC 2162	S. ser. Typhimurium ATCC 1408
TDLE	10±0^{hi}	10±0.58^{hi}	7.33±0.58^{kl}	7.33±2.31^{kl}
TDFH	15.67±2.08^{g}	15.75±1.15^{g}	7.33±1.15^{kl}	6±0^{l}
TDLM	11±1^{h}	11.5±58h	7.33±4.0.58^{kl}	11.67±0.58^{h}
TDLH	17.67±2.08^{f}	17±0^{f}	6±0^{l}	6±0^{l}
TDFE	18±2^{f}	18.5±0^{f}	6±0^{l}	6.67±0.58^{kl}
WURE	8.67±0.58^{jk}	6±0^{l}	6.67±0.58^{kl}	6.67±1.15^{kl}
WURH	14±1^{g}	8.33±0.58^{jkl}	6.67±1.15^{kl}	10.67±4.62^{hi}
WUSBE	6±0^{l}	7±0^{kl}	6.33±0.58^{kl}	6±0^{l}
WUSBH	11±3.21^{h}	7.33±2.31^{kl}	6.33±0.58^{kl}	7.33±0.58^{kl}
DMSO(-)	6±0^{l}	6±0^{l}	6±0^{l}	6±0^{l}
CHLO(+)	23.33±0.58^{de}	24±1.73^{cde}	24.33±0.58^{cde}	8.67±0.58^{jk}
CIPRO(+)	26±2^{abc}	23.33±2.52^{e}	26±0^{abc}	19.67±1.53^{f}

IZ = Inhibition zone (in mm) includes the diameter of the disc, TDLE = *Tithonia diversifolia* leaf extract of ethyl acetate, TDLH = *Tithonia diversifolia* leaf extract of hexane, TDLM = *Tithonia diversifolia* leaf extract of methanol, TDFH = *Tithonia diversifolia* flower extract of hexane, TDFE = *Tithonia diversifolia* flower extract of ethyl acetate, WURE = *Warburgia ugandensis* root extract of ethyl acetate, WURH = *Warburgia ugandensis* root extract of hexane, WUSBE = *Warburgia ugandensis* stem bark extract of ethyl acetate, WUSBH = *Warburgia ugandensis* stem bark extract of hexane, DMSO(-)=Dimethyl sulphur dioxide (Negative control), CIPRO(+) = Ciprofloxacin (Positive control), CHLO(+) = Chloramphenicol (Positive control), Values are means of triplicate readings (Means ± SD). Means followed by different superscript letters in the table above are significantly different at P < 0.0001.

respectively (Table 4). The extract showed MIC of 0.061 mg/ml against *S.enterica* ATCC 2162 with inhibition zone of 6 mm. The *T. diversifolia* extracts had anti-*Salmonella* activity against all the tested clinical isolates. Methanol extract of the leaves showed activity with MIC values of 0.031, 0.24, 0.448 and 0.98 mg/ml against *S.*ser.Typhi ATCC 43579, *S.* ser. Typhi ATCC 13347, *S.* ser. Typhimurium ATCC 1408 and *S. enterica* ATCC 2162 with inhibition zones of 11.5, 11, 11.67 and 7.33, respectively.

The nine active plant extracts were screened further to study the presence of medicinally active phytochemicals in leaves, stem barks, roots and flowers. Phytochemical analysis revealed presence of alkaloids, saponin, tannins, flavanoids, steroids, terpenoids and glycosides (Table 5). Steroids were detected in all the extracts. Flavonoids and tannins were absent in hexane extracts of *T. diversifolia* flower and *W. ugandensis* stem bark. Terpenoids were found in extracts of hexane and ethyl acetate from *T. diversifolia* flower, *W. ugandensis* root and *W. ugandensis* stem bark. Extracts of hexane (*T. diversifolia* flower) and (*W. ugandensis* stem bark) lacked alkaloids. Glycosides were detected in extracts of hexane and ethyl acetate (*T. diversifolia* leaf) and ethyl acetate (*W. ugandensis* stem bark). Saponins were detected only in extracts of methanol from *T. diversifolia* leaf (Table 5).

DISCUSION

In the present study, the extracts from 5 medicinal plants were screened for activity against clinical *Salmonella* strains. Of the extracts tested, both ethyl acetate and hexane extracts of *T. diversifolia* and *W. ugandensis* exhibited activity against all four *Salmonella* strains tested in this study. Methanol extracts of *T. diversifolia* leaf also inhibited all the clinical isolates tested.

T. diversifolia plant extracts exhibited different zones of inhibition against the isolates. The ethyl acetate flower and hexane leaf extracts of *T. diversifolia* gave zones of inhibition at 18.5 ± 5 mm and 17.67 ± 2 mm, respectively (Table 2). This compared well with ciprofloxacin which gave zone of inhibition of 19.67 mm and therefore no significant difference in activity (p < 0.0001). Methanol extract of *T. diversifolia* leaf also showed anti-Salmonella activity against test isolates. The observed anti salmonella activity of *T. diversifolia* extracts agrees with the finding of Ogunfolakan et al. (2010), on broad spectrum antimicrobial activity. Kareru et al. (2010) has reported that soap made from leaf extract of *T. diversifolia* was effective against *E. coli*.

The phytochemicals and secondary metabolites from plants possess antimicrobial activity (Srikumar et al., 2007). Phytochemical analysis demonstrated the presence of alkaloids, tannin, flavonoids, terpenoids steroids and glycosides in the active extracts. Ethyl acetate flower extracts exhibited the highest anti-*Salmonella* activity. This is due to the difference in the type and concentrations of secondary metabolites in different plant parts (Srikumar et al., 2007) and may be contributing to the observed differences in anti-*Salmonela*

Table 3. Minimum Inhibitory Concentration (mg/ml) of Hexane, ethyl acetate and methanol extracts

Plant extracts	*Salmonella* organisms			
	S.ser.Typhi ATCC 13347 mg/ml	S.ser.Typhi ATCC 43579 mg/ml	S.enterica ATCC 2162 mg/ml	S. ser. Typhimurium ATCC 1408 mg/ml
TDLE	0.24^{f}	0.061^{h}	0.031^{j}	0.98^{d}
TDFH	0.98^{d}	0.12^{g}	3.91^{b}	3.91^{b}
TDLM	0.24^{f}	0.031^{j}	0.98^{d}	0.488^{e}
TDLH	0.24^{f}	0.24^{f}	1.95^{c}	0.488^{e}
TDFE	0.98^{d}	15.63^{a}	0.12^{g}	3.91^{b}
WURE	0.24^{f}	0.031^{j}	0.061^{h}	0.12^{g}
WURH	0.031^{j}	0.031^{j}	3.91^{b}	0.031^{j}
WUSBE	0.031^{j}	0.031^{j}	0.061^{h}	0.031^{j}
WUSBH	0.031^{j}	0.031^{j}	0.488^{e}	0.0467^{i}
DMSO(-)	ND	ND	ND	ND
CHLO(+)	0.022^{klm}	0.029^{jk}	0.024^{jk}	0.030^{j}
CIPRO(+)	0.02^{lm}	0.015^{n}	0.018^{lm}	0.025^{jk}

ND =Not defined, TDLE=*Tithonia diversifolia* leaf extract of ethyl acetate,TDFH= *Tithonia diversifolia* flower extract of hexane, TDLM= *Tithonia diversifolia* leaf extract of methanol, TDLH= *Tithonia diversifolia* leaf extract of hexane, TDFE= *Tithonia diversifolia* flower extract of ethyl acetate, WURE=*Warburgia ugandensis* root extract of ethyl acetate, WURH= *Warburgia ugandensis* root extract of hexane, WUSBE= *Warburgia ugandensis* stem bark extract of ethyl acetate, WUSBH= *Warburgia ugandensis* stem bark extract of hexane, DMSO(-VE)=Dimethyl sulphur dioxide (Negative control), CIPRO(+VE)=Ciprofloxacin(Positive control), CHLO(+VE)=Chloramphenicol(Positive control), Values are means of duplicate readings. Means followed by different superscript letters in the table above are significantly different at P<0.0001.

activity. The results of this study show that extract with high content of steroids contribute to significant inhibition of *Salmonella* growth. This finding agrees with Ashok and Vijayalakshmi (2013) who have demonstrated that sterols from *Vitis vinifera* seed exhibited antibacterial activity. The sterols could be interacting with the bacterial cell wall and membrane ultimately leading to pore formation and disrupting bacterial membrane integrity (Devjani and Barkha, 2011). Odeyemi et al. (2014) has also reported that *T. diversifolia* leaf, flower and roots extracts have antibacterial activity due to the presence of metabolic toxins such as flavonoids, steroids and alkaloids.

Hexane extracts of *W. ugandensis* roots and stem barks showed inhibition zones of 14 and 11 mm, respectively against *S.*ser.Typhi ATCC 13347. Hexane extract of *W. ugandensis* roots and chloramphenicol showed inhibition zones of 10.67 and 8.67 mm against *S.* ser. Typhimurium ATCC 1408, respectively (Table 2). This was significantly lower than that of ciprofloxacin (19.67 mm). The observed anti-*Salmonella* activity of *W. ugandensis* is however supported by Yibeltal et al. (2013) who demonstrated activity of crude and semi-purified fractions of *W. ugandensis* against *Shigella boydii* and *Staphylococcus aureus*. Studies carried out by Olila et al. (2001) on aqueous extracts of *W. ugandensis* stem bark showed activity against both *Escherischia coli* and *S. aureus* in agar well assays but not in disc diffusion assay. The anti-*Salmonella* activity of *W. ugandensis* observed in our present study could be attributed to several secondary metabolites, among them steroids.

The extracts of three plants namely *Croton megalocarpus*, *Croton edulis* and *Lactoria cornuta* had no anti-*Salmonella* activity for the extracts (Table 2). Their anti-*Salmonella* activity values were not significantly different with those of negative controls (p < 0.0001). The lack of anti-*Salmonella* activity in these plants may not necessarily imply the same *in vivo* since compounds may either act as pro-drug which must undergo metabolic changes to achieve the required activity. Besides, the presence of bioactive compounds depends on many factors such as the season, age, intra-species variation, part of the plant collected, soil and climate (Gessler et al., 1995).

The MIC values of the nine active plant extracts was determined. These extracts were selected because of their appreciable anti-*Salmonella* performance determined by disc diffusion. The active extracts against *Salmonella* strains were *W. ugandensis* bark, *W. ugandensis* root, *T. diversifolia* leaf and *T. diversifolia* flower. The ethyl acetate extract of *W. ugandensis* stem bark showed anti-*Salmonella* activity among the extracts tested. The extract had MIC value of 0.031 mg/ml

Table 4. Mean anti-Salmonella activity values obtained by disc diffusion and microdilution technique for the active plant extracts against Salmonella strains.

Plant extracts		Salmonella organisms			
		S.ser.Typhi ATCC 13347	S.ser.Typhi ATCC 43579	S.enterica ATCC 2162	S. ser. Typhimurium ATCC 1408
TDLE	MIC (mg/ml)	0.24	0.061	0.031	0.98
	IZ (mm)	10±0	10±0.58	7.33±0.58	7.33±2.31
TDFH	MIC (mg/ml)	0.98	0.12	3.91	3.91
	IZ (mm)	15.67±2.08	15.75±1.15	7.33±1.15	6±0
TDLM	MIC (mg/ml)	0.24	0.031	0.98	0.488
	IZ (mm)	11±1	11.5±0.58	7.33±1.15	11.67±0.58
TDLH	MIC (mg/ml)	0.24	0.24	1.95	0.488
	IZ (mm)	17.67±2.08	17±0	6±0	6.67±0.58
TDFE	MIC (mg/ml)	0.98	15.63	0.12	3.91
	IZ (mm)	18±2	18.5±0	6±0	6.67±0.58
WURE	MIC (mg/ml)	0.24	0.031	0.061	0.12
	IZ (mm)	8.67±0.58	6±0	6.67±0.58	6.67±1.15
WURH	MIC (mg/ml)	0.031	0.031	3.91	0.031
	IZ (mm)	14±1	8.33±0.58	6.67±1.15	10.67±4.62
WUSBE	MIC (mg/ml)	0.031	0.031	0.061	0.031
	IZ (mm)	6±0	7±0	6.33±0.58	6±0
WUSBH	MIC (mg/ml)	0.031	0.031	0.488	0.0457
	IZ (mm)	11±3.2	7.33±2.31	6.33±0.58	7.33±0.58
DMSO(-)	MIC (mg/ml)	ND	ND	ND	ND
	IZ (mm)	6±0	6±0	6±0	6±0
CHLO(+)	MIC (mg/ml)	0.022	0.029	0.024	0.030
	IZ (mm)	23.33±0.58	24±1.73	24.33±0.58	8.67±1.53
CIPRO(+)	MIC (mg/ml)	0.02	0.015	0.018	0.025
	IZ (mm)	26±2	23.33±2.52	26±0	19.67±1.53

MIC= minimum inhibitory concentration (mg/ml), IZ=Inhibition zones (mm), TDLE=*Tithonia diversifolia* leaf extract of ethyl acetate, TDFH= *Tithonia diversifolia* flower extract of hexane, TDLM= *Tithonia diversifolia* leaf extract of methanol, TDLH= *Tithonia diversifolia* leaf extract of hexane, TDFE= *Tithonia diversifolia* flower extract of ethyl acetate, WURE=*Warburgia ugandensis* root extract of ethyl acetate, WURH= *Warburgia ugandensis* root extract of hexane, WUSBE= *Warburgia ugandensis* stem bark extract of ethyl acetate, WUSBH= *Warburgia ugandensis* stem bark extract of hexane, and CMBM= *Croton megalocarpus* bark extract of methanol, DMSO (-VE)=Dimethyl sulphur dioxide (Negative control), CIPRO(+VE)=Ciprofloxacin(Positive control), CHLO(+VE)=Chloramphenicol(Positive control).

against *S.*ser.Typhi ATCC 13347, *S.*ser.Typhi ATCC 43579, and *S.*ser.Typhimurium ATCC 1408. It showed MIC value of 0.061 mg/ml against *S. enterica* ATCC 2162. Hexane extract of *W. ugandensis* root showed MIC value of 0.031 mg/ml against *Salmonella* strains tested except *S. enterica* ATCC 2162 which was inhibited at 3.91 mg/ml. The extracts of hexane (stem bark) and ethyl acetate (root) from *W. ugandensis* showed anti-Salmonella

Table 6. Phytochemical constituents of the active plant extracts.

Plant extracts	Alkaloids	Saponin	Tannins	Flavanoids	Steroids	Terpenoids	Glycosides
TDLE	+	-	+	+	+++	-	+
TDFH	-	-	-	-	+++	+	-
TDLM	+	++	++	+	+++	-	-
TDLH	+	-	++	+	+++	-	+
TDFE	+	-	+	+	+++	++	-
WURE	+	-	+	+	++	++	-
WURH	-	-	+	+	++	++	-
WUSBE	+	-	+	+	++	+	++
WUSBH	-	-	-	-	++	++	-

- = absent; + = present; ++ = Moderate concentration; +++ = High concentration. TDLE=*Tithonia diversifolia* leaf extract of ethyl acetate, TDFH= *Tithonia diversifolia* flower extract of hexane, TDLM= *Tithonia diversifolia* leaf extract of methanol, TDLH= *Tithonia diversifolia* leaf extract of hexane, TDFE= *Tithonia diversifolia* flower extract of ethyl acetate; WURE=*Warburgia ugandensis* root extract of ethyl acetate, WURH= *Warburgia ugandensis* root extract of hexane, WUSBE= *Warburgia ugandensis* stem bark extract of ethyl acetate, WUSBH= *Warburgia ugandensis* stem bark extract of hexane, and CMBM= *Croton megalocarpus* bark extract of methanol.

salmonella activity against all strains tested (Table 3). Anti-*Salmonella* activity of *W. ugandensis* extracts compared well with ciprofloxacin and chloramphenicol. In a study carried out by Yibeltal et al. (2013), on antimicrobial activity of crude extracts of *W. ugandensis* against *E. coli* and *P. aeruginosa* demonstrated MIC values of 1.75 mg/ml. These results compared well with those of our study, which were in the range of 0.031 to 3.91 mg/ml (Table 3).

The methanol extract of *T. diversifolia* leaf had MIC values ranging from 0.031 to 0.98 mg/ml. The extract gave MIC values of 0.031, 0.24, 0.98 and 0.488 against *S.*ser.Typhi ATCC 43579, *S.*ser.Typhi ATCC 13347, *S.*ser.Typhimurium ATCC 1408 and *S. enterica* ATCC 2162, respectively (Table 3). Methanol and ethyl acetate extracts of *T. diversifolia* exhibited MIC values of 0.031 mg/ml each against *S.*ser.Typhi ATCC 43579 and *S.enterica* ATCC 2162, respectively. These values were not significantly different from ethyl acetate extracts of *W.ugandensis* ($p < 0.0001$). It was noted in our study that clinical *Salmonella* strains were sensitive to all *T. diversifolia* extracts at different MIC values (Table 3). Our present study has demonstrated lower MIC values for *T. diversifolia* extracts against *Salmonella* strains than what Ogundare (2007) reported. According to their report, MIC values of chloroform and methanol extracts of *T. diversifolia* were 6.25 and 3.125 mg/ml, respectively against *S. typhi*. The two extracts however gave MIC values of 6.25 mg/ml each against *P. aeruginosa*.

It was noted from this study that plant extracts tested by microdilution technique showed higher anti-*Salmonella* activity compared to values obtained from disc diffusion technique. *W. ugandensis* extracts showed lower MIC values when determined by microdilution method than by disc diffusion method. Olila et al. (2001) has reported that the paper disc retains the active component and does not allow it to diffuse into Muller Hinton agar. The paper disc is composed of cellulose [D-(1-4) linked glucose monomers]. The many free hydroxyls groups present on each glucose residues renders the surface of hydrophilic (Burgess et al., 1999). Thus, if natural products were cationic, they would be expected to adsorb to the surface of the disc and not diffuse into the medium. Consequently, a cationic polar compound displays a good antibacterial activity, but which is therefore not noticeably antibacterial by paper disc diffusion (Cleidson et al., 2007).

Most of the antibiotics used nowadays have lost their effectiveness due to development of resistant genes in microbes (Davis, 1994; Service, 1995). The antibiotics are sometimes associated with side effects such as hypersensitivity, immune suppression and allergic reaction (Ahmad et al., 1998). More interest is being shown in developing alternative antimicrobial drugs for the treatment of infectious diseases without side effects (Berahou et al., 2007; Salomao et al., 2008). The results of our present study demonstrates anti-*Salmonella* activity of *W. ugandensis* and *T diversifolia* that compared well with ciprofloxacin and chloramphenicol. The results obtained from the nine active plant extracts tested are encouraging. Further work is in progress to isolate and identify the bioactive compound(s) that could be used in the development of safer and cost effective alternative drugs for typhoid fever.

Conclusion

The results of our study showed anti-*Salmonella* activity in extracts from *W. ugandensis* and *T. diversifolia* plants. This activity compared well with that of ciprofloxacin and chloramphenicol. The study provides the basis for use of these plants in the development of drugs for management of typhoid fever.

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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Activity of Semi Purified Fractions of *T. diversifolia* and *W.ugandensis* against Selected Clinical Isolates of *Salmonella* Strains

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Abstract: The aim of this study was to determine *in vitro* anti-*Salmonella* activity of semi purified fractions of methanol extract of *Tithonia diversifolia* leaves, ethyl acetate and hexane extracts of *Warburgia ugandensis* stem bark and roots against four clinical isolates of *Salmonella* strains. The methanol, ethyl acetate and hexane extracts of the two plants were purified using silica column chromatography. Minimum Inhibitory Concentrations (MICs) of the semi purified fractions determined by microdilution assay. The MIC values of the fractions ranged from 1.22-312.5μg/ml. These results were comparable with that of ciprofloxacin (1.22-19.53μg/ml). Gas Chromatography-Mass Spectrometry (GC-MS) analysis was carried out to identify the important compounds in the active fractions. A total of thirty three known compounds were identified by GC-MS analysis. For example, hexadecanoic acid, 9, 12-octadecadienoic acid (Z, Z), 1, 2-benzenedicarboxylic acid and beta-sesquiphellandrene identified by GC-MS are known to have antimicrobial property. These findings demonstrate that the semi purified fractions of *T.diversifolia* and *W. ugandensis* are diverse and exhibit appreciable amount of anti-*Salmonella* activity and thus have great potential as a source for natural health products.
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Keywords: *T. diversifolia, W. ugandesis*, Anti-*Salmonella* activity, Microdilution assay, Silica gel column chromatography, GC-MS

1. Introduction

Salmonella serotype Typhimurium (*S.* ser. Typhimurium), is a Gram-negative bacterial pathogen that infects humans and animals, causing significant morbidity and mortality worldwide (Fink and Cookson, 2007). It is an obligate intracellular bacterial pathogen that causes gastroenteritis in millions of people worldwide each year (Grassl, *et al.*, 2008). For instance, the Centre for Disease Control (CDC) estimates that there are nearly 1.4 million food-borne *Salmonella* infections annually in the USA (Mead, *et al.*, 1999). Various strategies have been employed in the treatment and management of *Salmonella* infections.

Fluoroquinolones and tetracyclines are most commonly used to treat *Salmonella* infections. However, *Salmonella* strains resistant to these antibiotics have been reported in Korea and other countries (Choi *et al.*, 2005, and Stevenson *et al.*, 2007). One major concern to public health has been the global dissemination of *S.*typhimurium Definitive Type 104, which is resistant to cotrimoxazole, nalidixic acid and ampicilin (Parron *et al.*, 2008, Kariuki *et al.*, 2010). The rise in antibiotic-resistant strains has led to increased interest in use of plant materials to develop new effective drugs. Hence, there is a need for scientific evidence based validation of bioactive phytochemicals (Adeniyi and Ayepola, 2008; Karim *et al.*, 2011).

Plants used in this study have traditionally been associated with disease curative and preventive practices in many countries for a long time. Garcia and Delgado, (2006), have reported that *Tithornia diversifolia* has promising medicinal value. Skin products formulated from *T. diversifolia* extracts have been shown to have antimicrobial properties (Kareru *et al.*, 2010).

In Ethiopia *Warburgia ugandensis* extracts are used to treat malaria, tuberculosis, bronchitis, pneumonia, hepatitis, tapeworm, gonorrhea, and asthma (Wube *et al.*, 2010, Were *et al.*, 2010 and Opiyo *et al.*, 2011).

In the present study, *in vitro* bioassay guided purification of anti-*Salmonella* compounds from leaf of *T. diversifolia*; stem bark and root of *W. ugandensis* were carried out. The active fractions were identified by Gas Chromatography-mass spectrometry (GC-MS) analysis.

2. Materials and Methods

2.1 Salmonella strains

Clinical samples of *S.*ser.Typhi (ATCC 13347), *S.*ser.Typhi (ATCC 43579), *S.enterica* (ATCC 2162) and *S.* ser. Typhimurium (ATCC 1408), were provided by the Centre of Microbiology Research, Kenya Medical Research Institute (CMR-KEMRI) for this study.

2.2 Plant Materials

Methanol extract of *T.diversifolia* leaf, ethyl acetate and hexane extracts of stem bark and root respectively of *W.ugandensis* were obtained from the previous research work (Ogoti *et al*., 2015).

2.3 Controls

Acetone was used as negative control and Ciprofloxacin (Transchem pharmaceutical Ltd, Kenya) was used as positive control.

2.4 Silica gel column chromatography

A 60 cm long glass column with the diameter of 10 cm was filled with 1.5 kg of silica gel, mesh size 60-120. Methanol extracts of *T.diversifolia* leaf, ethyl acetate extracts of *W.ugandensis* bark and hexane extracts of *W.ugandensis* root were subjected separately to column chromatography in silica gel glass column. The column was eluted with hexane followed by hexane-ethyl acetate at increasing polarity. Fifty methanol, 20 ethyl acetate and 100 hexane fractions of 50ml each were collected, analyzed on TLC (Merck, S 0,032-0,063mm) with dichloromethane, chloroform and ethyl acetate solvents (3:2:1). The spots with similar RF values of methanol, ethyl acetate and hexane fractions were pooled to give 7, 4 and 7 sub-fractions of *T.diversifolia* leaf, *W.ugandensis* stem bark and root respectively. Various sub-fractions collected and labeled as documented in the Table 1, 2 and 3. All the obtained sub-fractions were collected in sample vials and stored at -20°C.

2.5 Minimum inhibitory concentration (MIC) values of semi purified fractions

The MIC values were determined using microdilution assay as described by Eloff (1998). Ciprofloxacin was used as positive controls and acetone was used as negative control. Plant fractions were tested against *Salmonella* strains with varying concentration ranging from 2.5mg/ml-0.0012mg/ml. Briefly, 100 µl of sterile distilled water was added to each well of 96-well microtitre plates (SIGMA Aldrich, German) followed by the addition of 100 µl of 2.5mg/ml and thereafter serially diluted plant fractions. Then 100 µl of *Salmonella* strains were added to each micro well to give a final volume of 200. The prepared plates were sealed to avoid drying and incubated overnight at 37□C. After overnight incubation, 50µl of 5mg/ml 2, 3, 5 Triphenyltetrazolium chloride (SIGMA Aldrich, German) was added to the wells and incubated overnight. The pink colour was indicative of bacterial growth while lack of color was linked to growth inhibition. The MIC was defined as the lowest concentration of plant fraction that completely suppresses the growth of *Salmonella* strains.

2.6 Gas Chromatography Mass Spectra

Gas chromatography-MS analysis GC-MS analysis was performed in Jomo Kenyatta University of Agriculture and Technology, Juja, Kenya. About 2 ml of methanol, ethyl acetate and hexane fractions were subjected to GC-MS analysis using CE GC 8000 top MSMD 8000 Fyson instrument with Db 35 mr column (10 m x 0.5 mm, 0.25 mm film thickness). Analysis was done at between 100-250°C for 3 minutes a flow rate maintained at 1ml/min in the split mode (1:50) (An aliquot (2 ml) of oil was injected into the column with the injector heater at 250°C). Analytical conditions Injection temperature at 250°C, interface temperature at 200°C, quadruple temperature at 150°C and ion source temperature at 230°C were maintained.

2.7 Identification of major components

The mass spectra of compounds in samples were obtained by electron ionization (EI) at 70 eV, and the detector operated in scan mode from 20 to 600 atomic mass units (amu). Identification was based on the molecular structure, molecular mass and calculated fragments. The spectrum of the unknown component was compared with the spectrum of the component stored in the NIST library version (2005), software, Turbomas 5.2.

2.8 Statistical Analysis

Anti-*Salmonella* activity was determined from means of duplicates in MICs. Collected data was analysed statistically using one way ANOVA (SAS, Version 9.0). Difference in values at P<0.0001 were considered statistically significant.

3. Results

3.1 Chromatographic fractionation of plant extracts

Methanol extracts of *T.diversifolia* leaf, ethyl acetate extracts of *W.ugandensis* bark and hexane extracts of *W.ugandensis* root were fractionated separately on silica column. Seven methanol fractions of *T.diversifolia* leaf designated T_A, T_B, T_C, T_D, T_E, T_F and T_G were obtained after silica TLC analysis. Likewise four ethyl acetate fractions of *W.ugandensis* bark designated E_A, E_B, E_C and E_D were determined by silica TLC analysis. Meanwhile, seven hexane fractions of *W.ugandensis* root designated H_A, H_B, H_C, H_D, H_E, H_F and H_G were also obtained by TLC analysis. All fractions were evaluated for anti-*Salmonella* activity by microdilution assay.

3.2 Anti-*Salmonella* activity of semi purified fractions of plant extracts

Eighteen semi purified fractions from methanol extracts of *T.diversifolia* leaves, ethyl acetate stem bark and hexane root extracts of *W.ugandensis* were screened for anti-*Salmonella* activity against 4 clinical isolates of *Salmonella* strains; *S.*ser.Typhi (ATCC 13347), *S.*ser.Typhi (ATCC 43579), *S.enterica* (ATCC 2162) and *S.* ser. Typhimurium (ATCC 1408) using microdilution assay.

The seven methanol fractions (T_A, T_B, T_C, T_D, T_E, T_F, T_G) had Minimum inhibitory concentration (MIC) values in the range of 1.22-312.5μg/ml, for the 4 clinical isolates of *Salmonella* strains. Table 1 shows MIC values of methanol fractions against the selected clinical isolates.

Table 1. MIC (μg/ml) of methanol fractions of *T.diversifolia* leaf

Plant fractions	Clinical isolates of *Salmonella* strains			
	S. ser. Typhimurium (ATCC 1408)	*S.*ser.Typhi (ATCC 13347)	*S.*ser.Typhi (ATCC 43579)	*S.enterica* (ATCC 2162)
T_A	9.77^{d}	2.44^{d}	4.88^{d}	4.88^{d}
T_B	312.5^{a}	1.22^{d}	19.53^{d}	1.22^{d}
T_C	78.13^{cd}	1.22^{d}	78.13^{cd}	1.22^{d}
T_D	39.06^{d}	1.22^{d}	19.53^{d}	39.06^{d}
T_E	39.06^{d}	1.22^{d}	19.53^{d}	39.06^{d}
T_F	39.06^{d}	1.22^{d}	78.13^{cd}	312.5^{a}
T_G	19.53^{d}	4.88^{d}	78.13^{cd}	2.44^{d}
CIPRO	19.53^{d}	1.22^{d}	9.77^{d}	1.22^{d}
Acetone	ND	ND	ND	ND

T: *T.diversifolia* leaf, T_A: Combined fractional 1-10, T_B: Combined fractions 11-17, T_C: Combined fractions 18-24, T_D: Combined fractions 25-32, T_E: Combined fractions 33-39, T_F: Combined fractions 40-44 and T_G: Combined fractions 45-50, CIPRO: Ciprofloxacin (Positive control) Acetone: Negative control, ND: Not determined. Values are means of duplicate reading. Means followed by different superscript letters in the table above are significantly different at P<0.0001.

Table 2 shows four ethyl acetate fractions with MIC values ranged from 1.22 to 312.5 μg/ml against the clinical isolates of *Salmonella* strains. All the fractions tested had anti-*Salmonella* activity.

Table 2. MIC (μg/ml) of fractions of ethyl acetate extracts of *W.ugandensis* stem bark

Plant Fractions	Clinical isolates of *Salmonella* strains			
	S. ser. Typhimurium (ATCC 1408)	*S.*ser.Typhi (ATCC 13347)	*S.*ser.Typhi (ATCC 43579)	*S.enterica* (ATCC 2162)
E_A	156.25^{bc}	19.53^{d}	156.25^{bc}	156.25^{bc}
E_B	9.77^{d}	2.44^{d}	312.5^{a}	9.77^{d}
E_C	1.22^{d}	1.22^{d}	19.53^{d}	1.22^{d}
E_D	1.22^{d}	1.22^{d}	9.77^{d}	1.22^{d}
CIPRO	19.53^{d}	1.22^{d}	9.77^{d}	1.22^{d}
Acetone	ND	ND	ND	ND

E: Ethyl acetate *W.ugandensis* stem bark, E_A: Combined fractions 1-6, E_B: Combined fractions 7-11, E_C: Combined fractions 12-16, E_D: Combined fractions 17-20. CIPRO: Ciprofloxacin (Positive control), Acetone: Negative control, ND: Not determined. Values are means of duplicate reading. Means followed by different superscript letters in the table above are significantly different at P<0.0001.

Table 3 shows seven hexane fractions of *W.ugandensis* roots that were obtained and evaluated for anti-*Salmonella* activity by microdilution assay. The MIC values of these fractions ranged from 1.22 to 312.5 μg/ml. It is evident from these results that *W. ugandensis* fractions had activity against all the *Salmonella* strains tested.

3.3 Identification of major compounds in fractions of methanol extracts of *T.diversifolia* leaves by GC-MS

The seven methanol fractions of *T.diversifolia* leaves designated T_A, T_B, T_C, T_D, T_E, T_F and T_G were subjected to GC-MS for identification of major compounds. Figure 1, 2, 3 and 4 are GC-MS chromatograms from analysis of fraction T_A, T_B, T_D and T_E respectively.

Retention times of 10.927 and 13.694 minutes are indicative of presence of, 2, 4-dimethylhexanoic and 3, 4, 5-trimethyl-1-hexene in fraction T_A. Other retention times were not linked to any known compound by GC-MS and were not identified in the NIST library database and need further exploration of the fraction to reveal identity

(Figure 1)

Table 3. MIC (µg/ml) of fractions of hexane extracts of *W.ugandensis* root

Plant Fractions	Clinical isolates of *Salmonella* strains			
	S. ser. Typhimurium (ATCC 1408)	*S.*ser.Typhi (ATCC 13347)	*S.*ser.Typhi (ATCC 43579)	*S.enterica* (ATCC 2162)
H_A	1.22^{d}	1.22^{d}	156.25^{bc}	312.5^{a}
H_B	1.22^{d}	1.22^{d}	78.13^{cd}	312.5^{a}
H_C	1.22^{d}	1.22^{d}	1.22^{d}	156.25^{bc}
H_D	39.06^{d}	1.22^{d}	1.22^{d}	1.22^{d}
H_E	19.53^{d}	312.5	156.25^{bc}	312.5^{a}
H_F	78.13^{cd}	1.22^{d}	1.22^{d}	9.77^{d}
H_G	78.13^{cd}	19.53^{d}	4.88^{d}	1.22^{d}
CIPRO	19.53^{d}	1.22^{d}	9.77^{d}	1.22^{d}
Acetone	ND	ND	ND	ND

H: Hexane *W.ugandensis* root, H_A: Combined fractions1-16, H_B: Combined fractions 17-23, H_C: Combined fractions 24-39, H_D: Combined fractions 40-58, H_E: Combined fractions 59-69, H_F: Combined fractions 70-89 and H_G: Combined fractions 90-1000, CIPRO: Ciprofloxacin (Positive control) Acetone: Negative control, ND: Not determined. Values are means of duplicate reading. Means followed by different superscript letters in the table above are significantly different at P<0.0001.

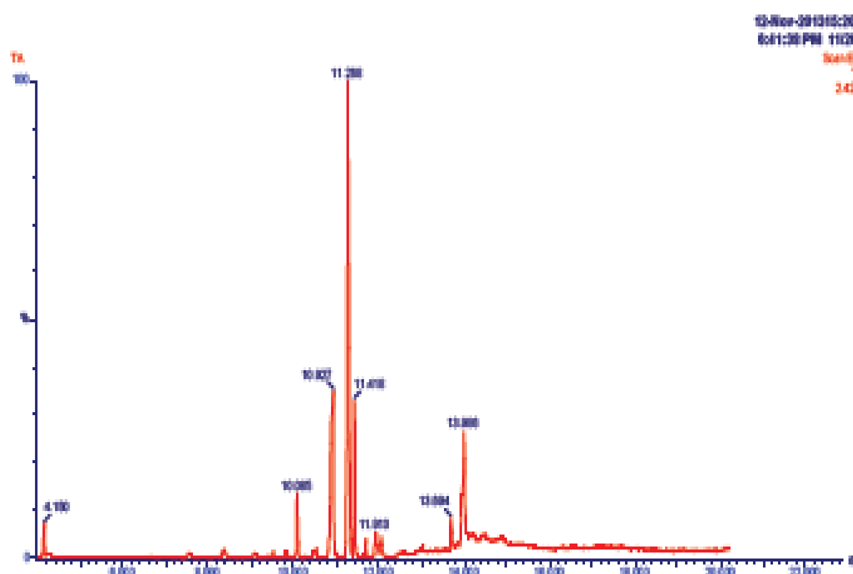

Figure 1. Chromatogram of T_A of methanol extract of *T.diversifolia*

In fraction T_B, peaks with retention times 10.927 and 11.994 were indicative of presence of n-hexadecanoic acid (palmitic acid) and 9, 12-octadecadienoic acid (Z, Z) compounds respectively. Other peaks in Figure 2 were not linked to any known compounds.

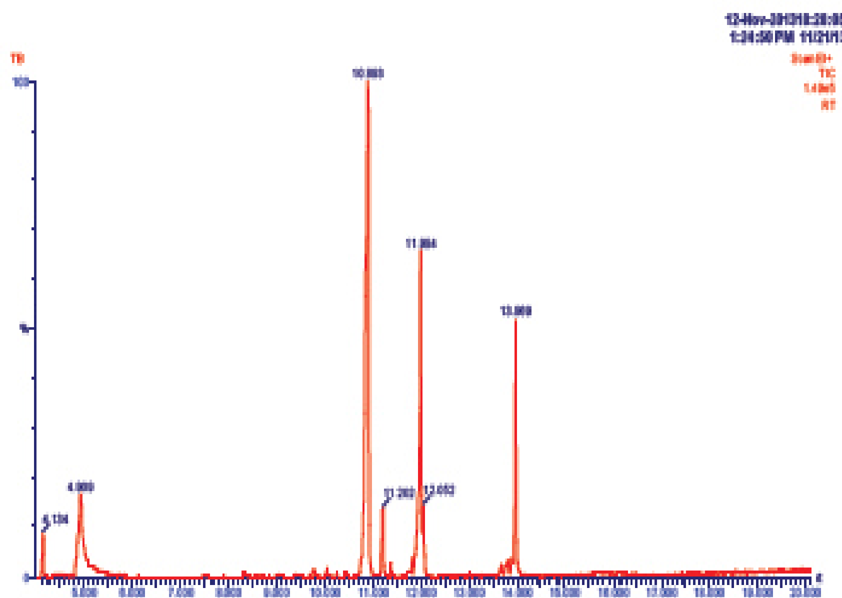

Figure 2: Chromatogram of T_B of methanol extract of *T.diversifolia*

In fraction T_D, peaks with retention times 5.344 and 9.376 were indicative of the presence 1, 2, 3-propanetriolmonoacetate and O-(2-methylpropyl) hydroxylamine compounds respectively (Figure 3).

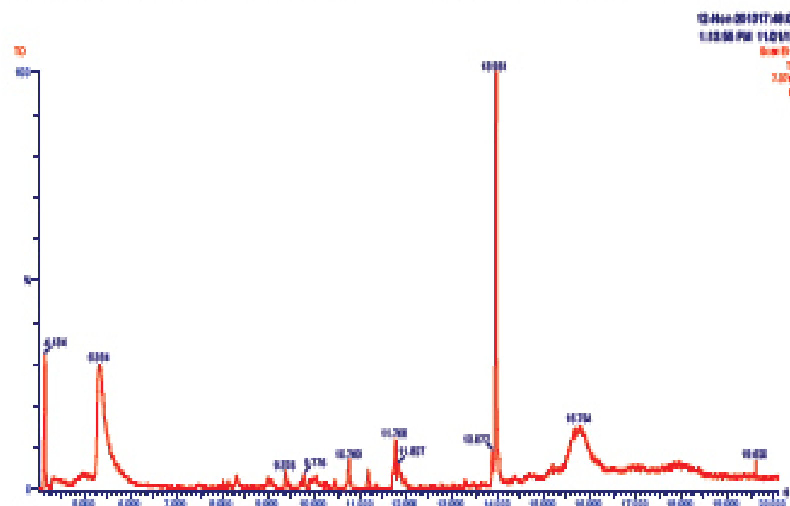

Figure 3. Chromatogram of T_D of methanol extract of *T.diversifolia*

In fraction T_E , peaks with retention times of 4.684, 9.318 and 15.086 are indicative of presence of, 1, 4:3,6-dianhydro – D- sorbitol, isorsorbide; E-2-Tetradecen-1-ol and Crotonic acid (o-formylphenylester compounds respectively (figure 4).

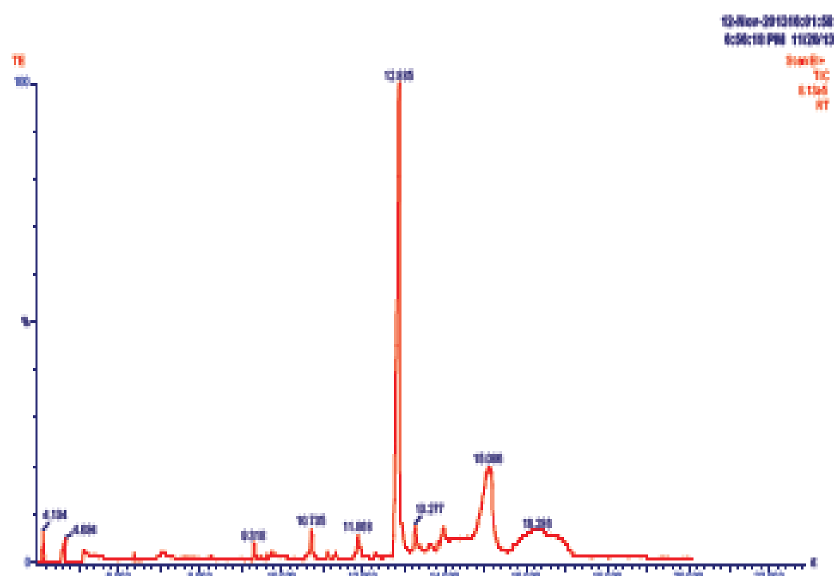

Figure 4. Chromatogram of T_E of methanol extract of *T.diversifolia*

Fraction T_F and T_G were not identified in the NIST library database and need further exploration of the fractions to reveal identity.

Table 4 illustrates the GC-Ms Retention Time (RT), molecular formula, and molecular weight of individual compounds of methanol fractions of *T. diversifolia* leaf.

Table 4. Components identified in methanol fraction of leaf of *T. diversifolia* by GC-MS analysis

Fraction	Peak	$T_{R(s)}$	M_F	M_W	Name of compound
T_A	1	10.927	$C_8H_{16}O_2$	144	2,4-dimethylhexanoic acid
	2	13.694	C_9H_{18}	126	3,4,5-trimethyl-1-hexene
T_B	1	10.893	$C_{16}H_{32}O_2$	256	n-hexadecanoic acid (palmitic acid)
	2	11.994	$C_{18}H_{32}O_2$	280	9,12-octadecadienoic acid(Z,Z)
T_C	---	---	---	---	Not determined
T_D	1	5.334	$C_5H_{10}O_4$	134	1,2,3-propanetriolmonoacetate
	2	9.376	$C_4H_{11}NO$		O-(2-methylpropyl)hydroxylamine
T_E	1	4.684	$C_6H_{10}O_4$	146	1,4:3,6-dianhydro – D- sorbitol, isosorbide
	2	9.318	$C_{14}H_{28}O$	212	E-2-Tetradecen-1-ol,Tetradecanal
	3	15.086	$C_{11}H_{10}O_3$	190	Crotonic acid (o-formylphenylester)
T_F	---	---	---	---	Not determined
T_G	---	---	---	---	Not determined

$T_{R(s)}$: Retention time (seconds), M_F: Molecular formula and M_W: Molecular weight (grams)

3.4 Identification of major compounds in ethyl acetate extracts of *W.ugandensis* stem bark by GC-MS

The four ethyl acetate fractions of *W.ugandensis* stem bark designated E_A, E_B, E_C and E_D were subjected to GC-MS for identification of major compounds. Table 5 shows the active principles with their retention time (RT), molecular formula and molecular weight (MW) of the identified compounds in fractions E_A and E_D. Fractions E_B and E_C were not linked to any known compounds in the NIST library database and need further exploration to reveal identity.

Table 5. Components identified in Ethyl acetate fraction of stem bark of *W.ugandensis* by GC-MS analysis

Fraction	Peak	T_{RM}	M_F	MW	Name of compound
E_A	1	6.359	$C_{13}H_{28}$	184	6-ethyl-2-methyldecane
	2	7.543	$C_{15}H_{24}$	204	Bicyclo[7.2.0]undec-4-ene,4,11,11-trimethyl-8-methylene-
	3	7.801	$C_{15}H_{24}$	204	1,4,8-cycloundecatriene-2,6,6-carophylene
	4	9.235	$C_{15}H_{26}O$	222	1,6,10-Dodecantrien-3-ol, 3,7,11-trimethyl-(E)-
	5	10.393	Unknown	Unknown	3-ethenyl-3-methyl-2,1-(1-methylethenyl)-6-(1-methylethyl)cyclohexanol
	6	14.011	$C_{22}H_{42}O_4$	370	Hexanedioic acid, bis(2-ethylhexyl)ester
E_D	1	10.843	$C_{16}H_{32}O_2$	256	Hexadecanoic acid/Palmitic acid
	2	11.910	$C_{18}H_{28}O_4$	252	E-15-heptadecanal

T_{RM}: Retention time (seconds), M_F: Molecular formula and M_W: Molecular weight (grams)

3.5 Identification of major compounds in hexane extracts of *W.ugandensis* roots by GC-MS

The seven hexane fractions of *W.ugandensis* root designated H_A, H_B, H_C, H_D, H_E, H_F and H_G were subjected to GC-MS analysis. Table 7 shows retention time, molecular weight and molecular formula of known compounds identified in fraction H_A, H_B and H_F. Fractions; H_C, H_D, H_E and H_G were not identified in the NIST library database and need further exploration of these fractions to reveal identity.

Table 6. Components identified in hexane fraction of root of *W.ugandensis* by GC-MS analysis

Fraction	Peak	T_{RM}	M_F	M_W	Name of compound
H_A	1	5.884	$C_{13}H_{28}$	184	6-ethyl-2-methyldecane
	2	6.384	$C_{17}H_{28}O_2$	264	Nerolidyl acetate
	3	6.993	$C_{15}H_{24}$	204	Beta Sesquiphellandrene
	4	9.776	$C_{18}H_{36}O_4$	316	9,10-dihydroxyloctadecanoic acid
	5	11.218	$C_{17}H_{32}O$	252	E-15-heptadecanal
	6	13.894	$C_{24}H_{38}O_4$	390	1,2-benzenedicarboxylic acid, diiisooctyl,
	7	19.410	$C_{13}H_{18}O_2$	206	2-cylohexane-1-one,2,4,4-trimethyl-3-(3-oxo-butenyl)-
H_B	1	4.867	$C_{21}H_{44}$	296	Heptadecane,2,6,10,14-tetramethyl-
	2	7.734	$C_{15}H_{26}O$	242	(S,3E,7E)-α,α,4,8-Tetramethyl-3,7-cyclodecadiene-1-methanol
	3	9.151	$C_{15}H_{26}O$	242	(2,5,5,8a-Tetramethyl-1,4,4a,5,6,8,8a-octahydro-1-napthlenyl)methanol
	4	11.594	$C_{15}H_{24}$	234	Spiro[5,5]undec-2-ene,3,7,7trimethyl-11 methylene(chamigrene)
	5	11.836	$C_{28}H_{32}O_8$	456	Pentacyclo[9.1.0.0(2,4).0(5,7).0(8,10)]dodecane
	6	12.053	$C_{15}H_{22}O_2$	384	(5aS,9aS,9bR)-6,6,9a-Trimethyl-5,5a,6,7,8,9,9a,9b-octahydronaphtho[1,2-c]furan-1(3H)-one (drimenol)
H_F	1	11.677	$C_{17}H_{32}O$	252	E-15-Heptadecanal
	2	12.994	$C_{15}H_{26}O_3$	284	1,1,4,6-Tetramethyldecahydro-1H-cycloprop[e]azulene-4,5,6-triol
	3	13.902	$C_{24}H_{38}O_4$	390	1,2-benzenedicarboxylic acid,diisooctyl ester

T_{RM}: Retention time (seconds), M_F: Molecular formula and M_W: Molecular weight (grams)

4. Discussion

Methanol extract of *T.diversifolia* leaf, ethyl acetate extract and hexane extract of stem bark and root of *W.ugandensis* respectively were fractionated on silica column and fractions obtained were analyzed by silica TLC. The TLC results indicated that seven methanol fractions of *T.diversifolia* leaf designated T_A, T_B, T_C, T_D, T_E, T_F and T_G, four ethyl acetate fractions of *W.ugandensis* stem bark designated E_A, E_B, E_C and E_D and seven hexane fractions of *W.ugandensis* root designated H_A, H_B, H_C, H_D, H_E, H_F and H_G were collected. All fractions were evaluated for anti-*Salmonella* activity by microdilution assay.

The MICs values of the seven methanol fractions of *T.diversifolia*; T_A, T_B, T_C, T_D, T_E, T_F and T_G were in the range of 1.22-312.5μgml^{-1} against the 4 clinical isolates of *Salmonella* strains. The MIC values of T_A were 9.77, 2.44, 4.88 and 4.88 μg/ml against *S.* ser. Typhimurium (ATCC 1408), *S.*ser.Typhi (ATCC 13347), *S.*ser.Typhi (ATCC 43579), *S.enterica* (ATCC 2162) respectively. The MIC value as low as 2.44 μg/ml of a semi purified fraction against the clinical isolates of *Salmonella* strains is suggestive of good anti-*Salmonella* activity of the compounds of T_A. The MIC values of T_B were in the range of 1.22-312.5μg/ml against the four strains tested. The lowest MIC value noted for this fraction was 1.22 μg/ml against *S.*ser.Typhi (ATCC 13347) and *S.enterica* (ATCC 2162) whereas *S.* ser. Typhimurium (ATCC 1408) was the least sensitive with MIC value of 312.5 μg/ml. The MIC values for fraction T_C, T_D, T_E, T_F and T_G were in the range of 1.22-312.5 μg/ml. It was noted in our study that clinical *Salmonella* strains were sensitive to all methanol fractions of *T. diversifolia* at different MIC values. This compared well with ciprofloxacin broad spectrum antibiotics, which gave MIC values of 1.22 to 19.53 μg/ml and there was no significance difference in the activity observed ($P<0.0001$). The observed anti-*Salmonella* activity of *T. diversifolia* fractions agrees with the finding of Obafemi *et al*., (2006), on broad spectrum antimicrobial activity on germacranolide type sesquiterpene lactone from *Tithonia diversifolia* leaf extract (MICs = 15.6 – 62.5mg/ml for most strains of bacteria tested). Maffo *et al*., (2006), has also reported that tithoniaquinone A isolated from leaf of *T. diversifolia* showed strong antibacterial activity against the Gram-positive bacterium *Bacillus megaterium* and antifungal activity against *Microbotryumviolaceum*.

The MIC values of the four ethyl acetate fractions of *W.ugandensis* stem bark designated E_A, E_B, E_C and E_D ranged from 1.22 to 312.5 μg/ml. In the present study, fraction E_C and E_D showed remarkable anti-*Salmonella* activity of 1.22 μg/ml against *S.* ser. Typhimurium (ATCC 1408), *S.*ser.Typhi (ATCC 13347) and *S.enterica* (ATCC 2162). In addition, fraction E_C and E_D had MIC values of 19.53 and 9.77 μg/ml respectively against *S.*ser.Typhi (ATCC 43579). Similarly, fraction E_A and E_B also exhibited appreciable amount of anti-*Salmonella* activity. The fractions of ethyl acetate (stem bark) showed anti-*Salmonella* activity against all strains tested. Anti-*Salmonella* activity of *W.ugandensis* fractions compared well with standard drug, ciprofloxacin broad spectrum antibiotics and there was no significance difference in the observed activities ($P<0.0001$). The observed anti-*Salmonella* activity of *W.ugandensis* is however supported by Yibeltal *et al*., (2013) who demonstrated activity of crude and semi-purified fractions of *W.ugandensis* against *Shigella boydii* and *Staphylococcous aureus*. Studies carried out by Olila *et al*., (2001) on aqueous extracts of *W.ugandensis* stem bark showed activity against both *Escherichia coli* and *Staphylococcous aureus* in agar well assays but not in disc diffusion assay. The anti-*Salmonella* activity of *W.ugandensis* observed in our present study could be attributed to several secondary metabolites, among them steroids, terpenoids and glycosides. This was supported by Ogoti *et al*., (2015), who reported on the activity of secondary metabolites found in the crude extracts of *W.ugandensis* stem bark.

The MIC values of seven hexane fractions of *W.ugandensis* root were determined. Fractions designated H_A, H_B, H_C, H_D, H_E, H_F and H_G exhibited remarkable anti-*Salmonella* activity in the range of 1.22 to 312.5μgml^{-1} against the 4 clinical isolates of *Salmonella* strains. Fraction H_D showed higher anti-*Salmonella* activity against three of the four clinically isolated *Salmonella* strain. Fraction H_D had MIC value of 1.22 μg/ml against *S.*ser.Typhi (ATCC 13347), *S.* ser.Typhi (ATCC 43579), *S. enterica* (ATCC 2162). It also exhibited MIC value of 39.06μg/ml against *S.*ser.Typhimurium ATCC 1408. Fraction H_E demonstrated the least anti-*Salmonella* activity against three out of the four strains tested. The fraction had MIC values of 312.5, 156.25 and 312.5 μgml^{-1} against *S.*ser.Typhi (ATCC 13347), *S.*ser.Typhi (ATCC 43579), *S.enterica* (ATCC 2162) respectively. However, it showed MIC value of 19.53 μg/ml against *S.*ser.Typhimurium (ATCC 1408). The hexane fractions of *W.ugandensis* root showed appreciable amount of anti-*Salmonella* activity against all strains tested. *Anti-Salmonella* activity of *W.ugandensis* (root) fractions compared well with the activity of ciprofloxacin (1.22-19.53 μg/ml). The present study has demonstrated lower or equal MIC values for *W.ugandensis* fractions (root) against *Salmonella* strains tested that are comparable to those of ciprofloxacin broad spectrum antibiotics. Therefore, anti-*Salmonella* activity for the fractions and that of ciprofloxacin had no significant difference ($p<0.0001$).

Our present study has demonstrated lower MIC values for *W.ugandensis* fractions (root) against *Salmonella* strains tested than what Yibeltal, *et al*., (2013), reported. According to their report, MIC values of semi-purified fraction of petroleum ether extract of *W.ugandensis* (heartwood) against both *S.boydii* and *S.aureus* was 500 μg/ml (0.5mg/ml) and 1000 μg/ml (1mg/ml) against *E.coli*. In addition, *Candida albicans* had MIC value of 1000 μgml^{-1} for semi-purified fraction of petroleum ether extracts of *W.ugandensis* both the leaf and the heartwood

(Yibeltal, *et al*., 2013). Results of growth inhibitory activity exhibited on the clinical isolates of *Salmonella* strains by hexane fractions of *W.ugandensis* root indicated the plant contained anti-*Salmonella* agents which supported its use in the local treatment of typhoid fever.Therefore the observed anti-*Salmonella* activity of fractions of *W.ugandensis* root in our present study could be attributed to several secondary metabolites, among them steroids, terpenoids and glycosides. These phytochemical compounds have been demonstrated to have anti-*Salmonella* activity by Ogoti *et al*., (2015).

A total of eighteen semi purified fractions from methanol extracts of *T.diversifolia* leaf, ethyl acetate stem bark and hexane root extracts of *W.ugandensis* were analyzed by GC-MS technique to identify the major compounds. Our study showed the presence of alkenes, fatty acids and short chain unsaturated carboxylic acid in the active methanol, ethyl acetate and hexane fractions. Nine important compounds were identified in methanol fractions based on the database in the NIST library. For instance, 9, 12-octadecadienoic acid (Z, Z) and n-hexadecanoic acid identified by GC-MS analysis are fatty acids whereas 3, 4, 5-trimethyl-1-hexene detected was alkenes. Crotonic acid identify in fraction T_8 is a short chain unsaturated carboxylic acid, among others. Meanwhile, eight important compounds were detected in the ethyl acetate fractions of *W.ugandensis* stem barks. Some of them include; hexanedioic acid, bis (2-ethylhexyl) ester, Hexadecanoic acid and E-15-heptadecenal. Likewise sixteen important compounds were also successfully identified in the hexane fractions of *W.ugandensis* root. E-15-heptadecenal, 1, 2-benzenedicarboxylic acid and 6-ethyl-2-methyldecane were detected among others.

Majority of the phytoconstituens identified in methanol, ethyl acetate and hexane fractions are attributed with various biological activities. For example, hexadecanoic acid is a very common saturated fatty acid, known anti-inflammatory phytoconstituent as it is a phospholipase inhibitor (Aparna *et al*., 2012) and it's also known for its antibacterial activity (Manilal *et al*., 2009). The n-hexadecanoic acid is also a known fatty acid that possesses antioxidant, hypocholesterolemic, nematicide, pesticide and antiandrogenic activity (Duke, 2007). Likewise 9,12-octadecadienoic acid (Z,Z), is otherwise called as omega 6 fatty acids which are a family of pro-inflammatory, anti-inflammatory polyunsaturated fatty acid and antimicrobial agents (Marimuthu *et al*., 2014). The 1, 2-benzenedicarboxylic acid and beta-sesquiphellandrene have been shown to have antimicrobial property (Duke *et al*., 2007, Vukovic *et al*., 2007). E-15-Heptadecenal, an aldehyde was identified in both hexane and ethyl acetate extract of *W.ugandensis*, has been reported for antibacterial activity (Vinay *et al*., 2011).

5. Conclusion

This study confirms the presence of therapeutically potent anti-*Salmonella* compounds in the methanol fractions of *T.diversifolia* leaf, ethyl acetate and hexane fractions of stem bark and root of *W.ugandensis* respectively that could lead to development of antibiotics against typhoid fever. Further work is in progress to determine the effect of active compounds on dihydrofolate reductase.

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