Antimicrobial activities of *uvariodendron anisatum* fractions

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DECLARATION

Declaration by the student

This research project is my original work. It has not been presented for the award of any degree to any other institution. No part of this research project should be reproduced without my consent or that of Mount Kenya University.

Signature…… Date……..

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Declaration by the supervisor

This research project has been submitted with my approval as The Mount Kenya University Supervisor.

Signature…….. Date……..

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DEDICATION

To my father, Gideon Muguheli & my mother the late Dorothy Makungu for their relentless support through thick & thin ever since I joined school. To my supervisor Dr Jared M Onyancha who was a great source of knowledge and encouragement.

ACKNOWLEDGEMENT

To start with, I thank the almighty God. I extend my appreciation sincere gratitude to my supervisor, Dr Jared M Onyancha for his advice & encouragement while performing experiments in the laboratory.Mr Eric Odoyo, the head of Microbiology department at Kenya medical research institute (KEMRI) was also of great help.

Mr. Nzivo, Pharmacognosy laboratory technician at Mount Kenya University offered his assistance while I was preparing the sample extract. Last but not least I thank Mount Kenya University for according me the opportunity to undertake a Bachelor's degree in Pharmacy.

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ABSTRACT

Conventional antimicrobial agents available for treating infections caused by microorganisms pose a wide range of challenges which include antimicrobial resistance & severe side effects. In an attempt to develop potent drugs for the treatment of infectious diseases, antimicrobial agents in natural products have been widely researched. This research project aimed at establishing the antimicrobial effects of *Uvariodendron anisatum* Fractions .The Fractions were obtained through column chromatography from *Uvariodendron anisatum* root extract which was prepared by maceration. Antibacterial activity against *Escherichia coli* & *Staphylococcus aureus* was investigated via disk diffusion method. Results obtained did indicate that the Fractions possess antibacterial activity. It can therefore be concluded that the root extracts of *Uvariodendron anisatum* have Fractions of great ethno medical importance & can be used for treatment of infections caused by *Escherichia coli* & *Staphylococcus aureus*.It is thus recommended that bioassay guided techniques be utilized in search of the active extracts from this plant. Furthermore, there is need to establish profiles of in vivo antibacterial effects of the isolated Fractions.

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ABBREVIATION OF TERMS

DMSO	-	Dimethyl sulphoxide
PET ETHER	-	Petroleum ether
DCM	-	Dichloromethane
UV	-	Ultraviolet
mg	-	Milligrams
Fig	-	Figure
mL	-	Milliliters
mcg	-	Microgram
uL	-	Microliters
mm	-	Millimeters

CHAPTER ONE

INTRODUCTION

1.1 Background information

Globally, traditional medicines and medicinal plants are regaining their lost glory in maintenance of good health. it's worth noting that modern pharmacopoeia is comprised of not less than 25% of medicines extracted from plants and others, which are artificial equivalents based on model compounds obtained from plants. Demand for these medicinal plants as a mitigation to health issues has been accelerated by skyrocketing costs attributed to prescription drugs in eradication of ailments, sustenance of personal health as well as general wellbeing of an individual and the bio-outlook of emerging plant-extracted drugs (Kigen et al. 2013). The current growing acknowledgement of medicinal plants is attributable to a number of reasons including the paradigm shift in faith with regard to herbal medicines.

Since time immemorial, phytotherapy has gained more attention due to the ease in availability & affordability of medicinal plants. At the of inception modernization, human beings in most cases have shown and instilled a culture of dependence on plant-based medicines and other pharmaceutical related products. (Shazadi et al., 2010). A few decades ago, most organisms of bacterial nature have occasionally displayed increasing trend against existing antibacterial agents (Nascimento et al., 2000). Plant manufactured medicines have been in use since ancient times to treat several illnesses. Antibacterial propertied render them a reservoir of potent drugs (Srivastava et al., 2005). The adoption of Herbal medicines has always played a pivotal role in controlling and managing conditions like heart disorders, elated blood sugar, and several cancers (Mohanta et al., 2003). Some kinds of medicinal plants have been crucial in the manufacturing of various individual drugs or in combinations. In addition, medicinal plants are utilized a major of raw material for conventional medicines (Tahir and Khan, 2012).

There is a definite trend to adopt plant based products since allopathic medicines are mostly associated with serious side effects and resistance against antibiotics makes these drugs non potent. About 80% of the global population rely on medicinal plants to take care of basic health related issues this is largely associated with affordability and almost available in times of needs (Shazadi et al., 2010). These plants should thus be well investigated to better comprehend their safety, efficacy and general properties.

1.2 Problem statement

Given the fact that several herbal medicines have been used for ages to cure many diseases, there has been inadequate input of scientific studies to validate the healing claims and perhaps isolate the active drug components.

1.3 Justification

This research project aimed at investigating the antimicrobial effects of Uvariodendron anisatum fractions. The data obtained will be vital in validating the plant for use as well as elucidation of active compounds for drug development.

1.4 Objective of study

1.4.1 General research objective

The overall objective of the study was to investigate and evaluate the antimicrobial effects of *Uvariodendron anisatum* Fractions.

1.4.2 Specific research objectives

1. To separate the Fractions obtained from the root extracts of *Uvariodendron anisatum* using thin layer chromatography.

2. To find out the antibacterial activity of *Uvariodendron anisatum* Fractions against *Escherichia coli* and *Staphylococcus aureus.*

CHAPTER TWO

LITERATURE REVIEW

2.1 Plants used in management of infections.

The use of plants for therapeutic purposes by humans, can be traced back to the Middle Paleolithic Age, almost sixty thousand years ago, this is as per exist8ing fossil records

(Fabricant and Farnsworth 2001). Some of the substances that provided medicinal effects were oils of *Cupressuss empervirens* (cypress) & *Cedrus* species (cedar) and *Glycyrrhiza glabra* (licorice). *Commiphora* species otherwise known as myrrh and poppy juice, are currently utilized for treating ailments that range from colds, coughs to infections caused by parasites (Gurib-Fakim 2006). Health care in olden days entailed the use of leaves, flowers, stems, roots and even berries of herbs for their therapeutic or medicinal value. Previously, medicines of these nature assumed the form of unrefined drugs like powders, poultices, tinctures and other formulations of herbal origin (Balick and Cox, 1996). The understanding of particular plants utilized and the approaches for application in treating specific maladies were transferred to subsequent generations via oral history, later details about medicinal plants were ultimately published in herbals (Balick and Cox, 1996).

Ammi majus also known as bishop's weed is majorly used to treat skin diseases in particular psoriasis . It is also used in treatement of T-cell associated lymphomas (Gurib-Fakim, 2006). *Piliostigma reticulatum* is used in the treatment of small pox and leprosy. *Euclea divinorum* has proven to be efficacious in the treatment of skin diseases and gonorrhea. Diabetes can be treated by *Euclea undulata*. *Bolusanihus speciosus* is used in the treatment of tuberculosis. The root bark extract of *Vangueria infausta* exhibits antimalarial activity. *Artemisia afra* has antimicrobial activity and is used in the treatment of intestinal worms. *Combretum apiculatum* is used to treat bacterial infections. *Helichrysum cymosum* is used to treat wound infections and as well as malaria. *Warburgia salutaris* of the Canellaceae family is used to treat bacterial,fungal, protozoal and yeast infections. **S**yphilis and conjunctivitis can be treated by the plant *Combrelum woodii*

2.2 General uses of Medicinal Plants

Medicinal plants otherwise known as phytomedicinals remain the principal form of medicine in a vast majority of countries. Almost three quarters of human beings on planet earth primarily depend on plant products in their crude nature to satisfy their health care related needs (Barrett and Kieffer 2001). In developing counties, eighty percent of the population largely rely on herbal medicines to meet their basic health care requirements according to the World Health Organization. Majority of the plant materials are used while still fresh for easy extraction of valuable component from the plant as whole or its parts including barks, flowers, fruits, leaves or roots.

For woody forms, the major parts used are the bark and roots. Others such as ginger, cloves and coriander otherwise known as carminates usually are fresh additives (Rao and Arora 2004). For example, *Arciostaphylos uva-ursi,* bearberry, and *Vaccinium macrocarpon,* cranberry juice, has been posited in various phytotherapy manuals to be effective in urinary tract infections treatment on the other hand lemon balm species*(Melissa officinalis),* garlic *(Allium sativum)* and tea tree (*Melaleuca alternifolia)* can be termed as agents with a broad spectrum of antimicrobial activity.

.Extracts of certain plants with tremendous medicinal value include of *Albizia gummifera sten bark decoction* which are critical in managing venereal illnesses (Buvva and Van Staden 2006). The majority of plants used chiefly as traditional medicines are subjected to scientific research validat5ion by isolation compounds of bioactive value to be utilized directly.

2.3 Assays for antimicrobial activity

The approved technique adopted in most clinical microbial laboratories is Disk diffusion. It is used to determine antimicrobial sensitivity or susceptibility. It employs approved standards or bacteria and yeast testing. It entails agar plates being inoculated with a standardized bacterial test microorganism inoculum . A filter paper disc (6 mm) in diameter containing the test component at a desired specific concentration is placed on the agar surface. This is then followed by incubating Petri dishes under favorable conditions. The antimicrobial agent then diffuses into agar and if the microorganism is susceptible, its growth is inhibited around the

antimicrobial containing disc. The zone of inhibition diameters are then measured in mm using a Vanier caliper or a ruler. Qualitative results are provided by an antibiogram for categorizing bacteria as resistant, intermediate or susceptible. It is also used as a selection tool for therapeutic interventions.

Broth dilution method may be done on a micro or macro scale. It entails preparation of 2 folds dilution of the antimicrobial agent. If the volume of this equation is > 2 ml, it is considered as macro dilution. If the volume of this equation is < 2 ml, it is considered as micro dilution. For micro dilution, a 96 well micro titration plate is normally used. Each tube is inoculated with an inoculum prepared with similar medium after dilution of the standardized microbial suspension. After mixing, the tubes are later incubated under suitable conditions mostly with no agitation and conditions depend on the microorganism being tested.

Agar dilution technique entails incorporation of varying specific concentrations of the test substance into the molten agar in two serial folds dilutions then followed by inoculation on the agar plate surface. The technique is most appropriate for antifungal and anti-base susceptibility test. In the case of multiple isolates being subjected to the test against an individual compound then the technique is preferable for liquid medium bacterial growth.

2.4 Active components of plants extracts

The important medicinal effects obtained from plant materials generally result from combining secondary products found in plants. The chief secondary metabolites include alkaloids, phenols, steroids & tannins, which are synthesized and either deposited in all or specific plant parts (Joseph and Raj 2010). Typically, leaves are the advantageous sites for storage of desired compounds. Other parts that contain a considerable amount of active constituents are fruits. The juice can be orally administered so as to obtain the desired beneficial compounds. Roots, and aerial parts, flowers, seeds, stem barks, etc. are equally of great significance and can thus be extracted for beneficial

therapeutic components (Chan *el al.,* 2012). Plant secondary metabolites are vital as a basis for the manufacture of significant artificial compounds for example pharmaceuticals, cosmetics and lately nutraceuticals (Bourgaud *el al*., 2001). They are highly regarded as latent sources of novel drugs, antibiotics, insecticides as well as herbicides (Crozier et al., 2006). This is due to the fact their biological value and likely health importance comprising of antioxidant, antiaging, antimicrobial, anti-inflammatory anticancer and anti-atherosclerotic effects. Flavonoids display a broad spectrum of activity which entails anti-carcinogenic, antiviral, anti-inflammatory, anti-thrombogenic, anti-oxidant and anti-therogenic properties.

Flavonoids and tannins have been reported to have antidiarrheal activity through increasing electrolyte and water reabsorption (Palombo, 2006). Coumarins exhibit antimicrobial activity. They are used to treat coughs, diabetes, colds, sore throat, headache, gout, malaria and intestinal worms. Phenolic compounds have been reported to be strong antioxidants (Chew et al. 2009).

2.5 Plant families with known antimicrobial activity in Africa

Several plant families exhibit antimicrobial activities. Fabaceae family, *Bolusunthus specious*, is used in the treatment of tuberculosis. Rubiaceae family, *Vanguena mfausta* is used in the treatment of malaria. Asteraceae family, *Artemisia afra,* has proved beneficial in malaria treatment and intestinal worms eradication. Ranunculaceae family, *Knowltonia vesicatoria* has been demonstrated to have antimycobacterial activity and proved effective in treatment of tuberculosis, Rutaceae family, *Vepris uguenesis*, has been use traditionally in the treatment of malaria (The Royal Society of Chemistry, 2015).

Annonacea family constitutes about 129 genera and 2120 species of trees, shrubs and woody climbers mostly scattered throughout temperate and tropical regions of the world. Annonacea family plants are characterised by varied secondary metabolites which include alkaloids, *glycosides,* coumarins, saponins, phenols, triterpenes, flavonoids, fatty acids and volatile oils.

2.5.1 *Uvariodendron anisatum*

Uvariodendron anisatum is commonly known as Mutonga (Embu). This plant species is a forest shrub or small tree 6m tall with a grey brown smooth bark. Its long leaves are aromatic when crushed. Flowers are cream yellow, appearing on old wood. The fruits are cylindrical and constricted between the seeds, dark blue when ripe. A rare shrub, the plant is mainly distributed in the central and southern parts of Kenya and belongs to Annonaceae family.

2.5.2 Biological properties of *Uvariodendron anisatum*

Extracts of the roots and barks in their aqueous form have been proclaimed to show significant in-vitro antibacterial *activity* against *Staphylococcus aureus.* Root decoction is taken to ease labour pains or if the afterbirth is late.

2.5.3 Ethno botanical Information of *Uvariodendron anisatum*

In Kenya, its root decoction has been reported to possess ethno medical uses such as easing labour pains or if the afterbirth is late. The infusion is used as a sexual stimulant.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

This study was investigational in nature. The plant was collected from Kiang'ombe forest in Embu County. Methanol was used to obtain the root extract which was then later subjected to antibacterial activity testing carried out in the Biochemistry laboratory at Mount Kenya University.

3.2 Preparation of the plant material

The harvested plant material (roots) of *Uvariodendron anisatum* were properly dried and grounded for assays in the laboratory.

3.3 Extraction

The Extract was prepared by maceration method.

Dried and grounded roots of *Uvariodendron anisatum* were soaked in methanol for 48hours. Filtration was done and the filtrate obtained placed in the oven for drying. The extracts were decanted and filtered through Whattman filter paper and the macerate steeped in solvent again for 48 hours. The filtrate obtained was combined with the first filtrate. Total mass of the extract obtained was 4.12g. The extract was then finally preserved aseptically at 4^0c in an airtight bottle and placed in the refrigerator.

Procedure for packing the column

The clamped, clean column was rinsed with petroleum ether solvent. A clean piece of cotton wool was stuffed at the base of the column to avert loss of the stationary phase. 50g of silica gel (mesh 60:120), mixed with petroleum ether was fed into the column. Gentle tapping of the column allowed bubbles escape &Silica to rise. The slurry was mixed with a clean glass rod. 2.0g of the sample extract was put on top of the silica gel. A thin layer of sand was then added on top of the sample extract to help ensure a level Silica gel line on top of the column.

3.4 Solvents and Materials

3.4.1. Solvents

-Mobile phase solvents were; Petroleum ether and Dichloromethane. Methanol was used to wash the column. 950 Litres of Petroleum ether were used. 5 313 Litres of Dichloromethane and 536 L of methanol.

3.4.2. Materials

The Whattman filter paper No.l (Whatmann International Ltd, Maidstone, England). Pre-coated Aluminum plates (250 pm thick layer of normal Silica gel 60 GF254), Eppendorf tubes, Pipettes and petri dishes.

Positive control was 30mcg Ciprofloxacin and Negative control 0.1% DMSO.

3.4.3 Microorganisms

The microorganisms tested on master cultures included *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922,

3.4.4 Apparatus and equipment

Clean glass beakers, a conical flask and a funnel were used during extract preparation. Drying of the filtrate was achieved by use of the oven. Column chromatography was achieved by use clean glass column. Fractions were collected via clean glass test tubes. 30 clean sterilized glass petri dishes were used to culture the micro-organisms.

CHAPTER FOUR

RESULTS AND DISCUSSION

Thin layer chromatography of isolated compounds

Figure 1: Figure (a): Isolated compounds as visualized by the naked eye (E)

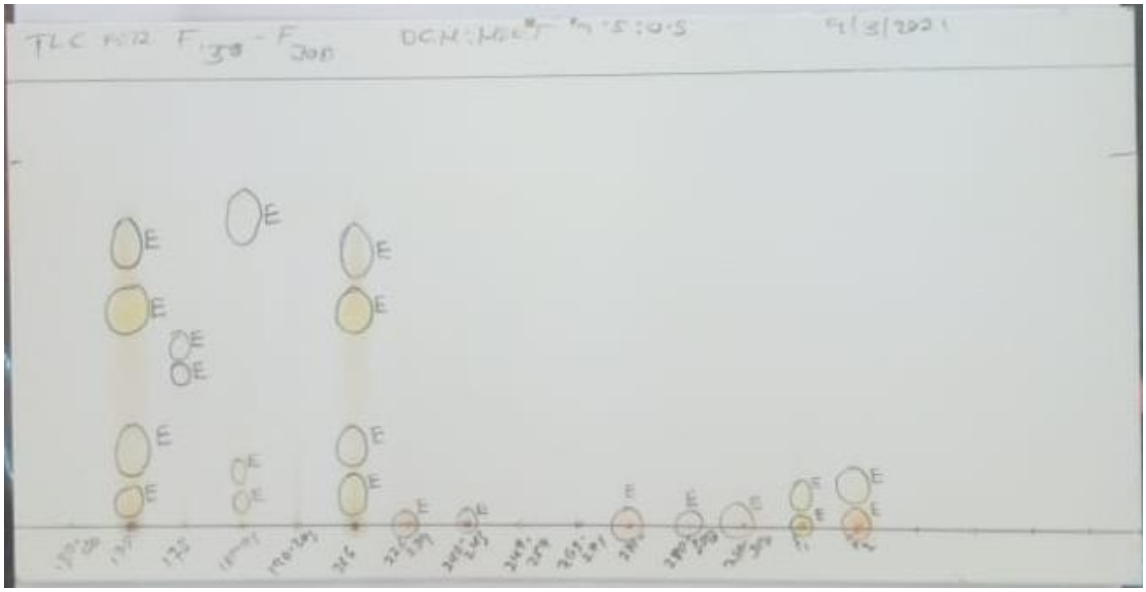

Figure 2: Figure (b): isolated compounds as visualized under short UV wavelength (254nm)

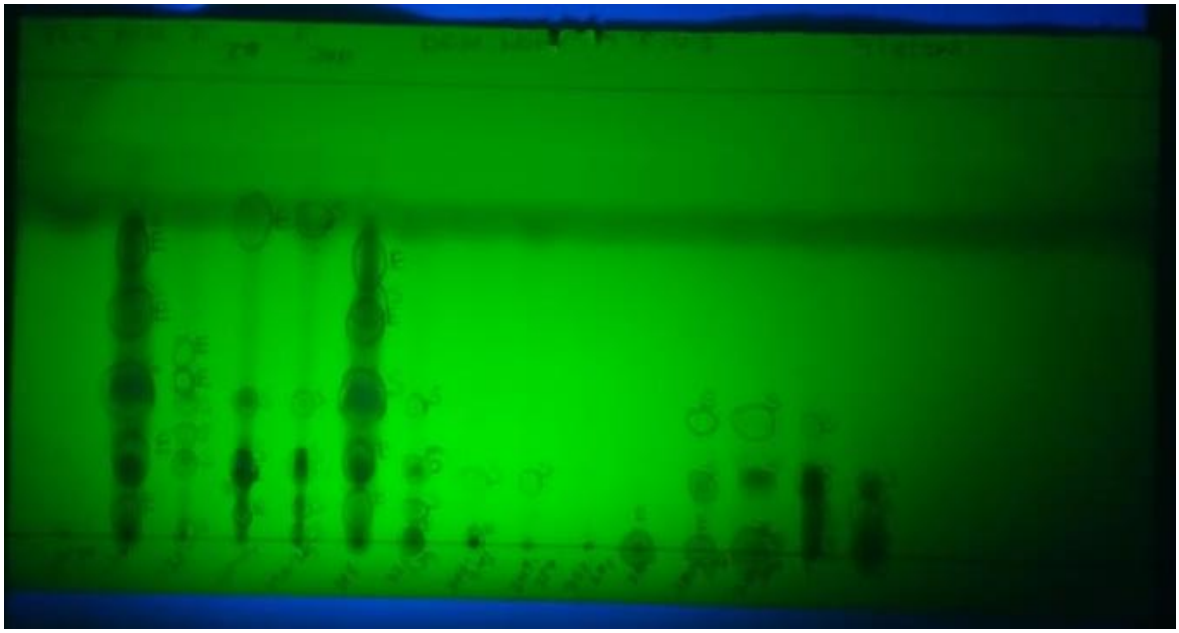

Figure 3: Figure (c): isolated compounds as visualized under long UV wavelength (365nm)

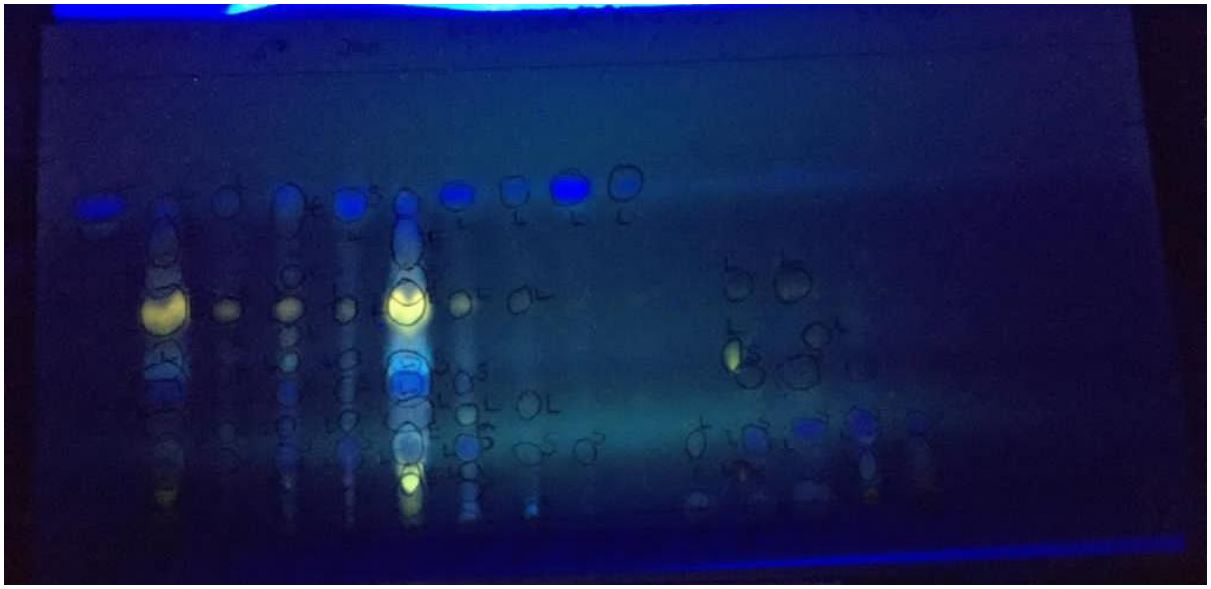

Figure 4: Figure (d): Isolated fractions as visualized under the naked eye, short wavelength & long wavelength

Figure 5: Figure (f): isolated compounds as visualized uder the naked eye,short wavelength,long wavelength & the iodine chamber (I)

DCM:MeOH (9.5:0.5)

Column Chromatography Results

A total of 380 fractions were collected. The first coloured fraction obtained was F177(light brown). Colour faded upon collection of fraction F199. Mobile phase was 1:9 Petroleum ether: Dichloromethane respectively.

Upon collection of fraction F216, a light green colour reappeared. The mobile phase was in the ratio 1:9 for Methanol & Dichlormethane respectively. Colour later on changed gradually & fractions F226-271 exhibited a light brown colour.Mobile phase was 1:1(50:50) Methanol: Dichloromethane respectively. From fractions F272-280,Mobile phase was ;Methanol: Dichloromethane (60:40) & 80:20 Methanol :Dichloromethane respectively for fractions F281-295. Fractions with similar Retardation factor values were combined together and this gave a total of six fractions; F1, F2, F130-150, F178, F216 & F226-239.

Antibacterial activity

Negative control: 0.1% DMSO

Positive control: 30mcg CIPROFLOXACIN

The table below shows the zones of inhibition at reducing concentration of each Fraction. 0.1% DMSO was used as the negative control and 30mcg Ciprofloxacin as the positive control.

Table 1: Antibacterial activity of the isolated Fractions

	Fraction	***Escherichia coli* (mm)**	***Staphylococcus aureus*)*mm***
1	F1	8	13
2	F2	10.8	8
3	130-150	8.3	7
4	178	7	7
5	216	12.5	8
6	226-239	7.3	9.5
7	Negative control	6	6
8	Positive control	36.5	30.5

Table 2: Graphical representation of antibacterial activity

Table 2: Graphical representation of antibacterial activity

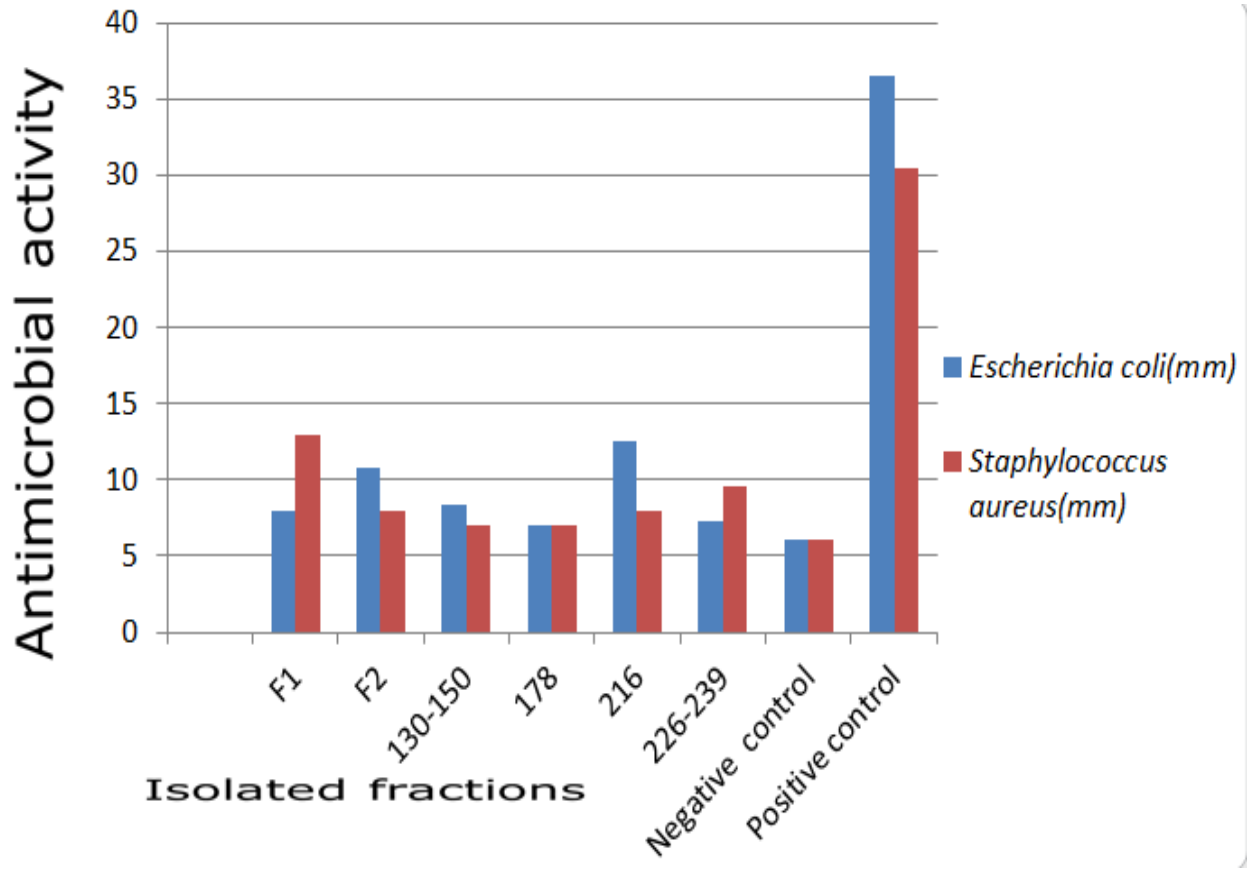

Table 3: Retardation Factor Values for Isolated fractions DCM: MOET 9.5: 0.5

	Fraction	*Escherichia coli* (mm)		*Staphylococcus aureus* (mm)	
		E	S	L	I
1	1	0.03, 0.09	0.03,0.09,0.18,0.34	0.03,0.09,0.18	0.29,0.49
2	2	0.05, 0.12	0.2	0.06,0.12,0.2	0.04,0.12,0.29,0.55
3	130-150		0.88	0.88,0.91	
4	178	0.73, 0.89,0.41	0.58,0.61,0.75	0.61,0.75,0.89	0.70
5	216	0.61, 0.78	0.42,0.61,0.78	0.55,0.61,0.78	
6	226-239		0.45	0.05,0.14,0.59,0.73,0.94	0.05,0.14,0.25

Table 4: Retardation Factor Values for Isolated fractions DCM: MOET 9:1

	Fraction	*Escherichia coli* (mm)		*Staphylococcus aureus* (mm)	
		E	S	L	I
1	1	0.23,0.39	0.22,0.28,0.39,0.45	0.03,0.58	0.11,0.28,0.53
2	2	0.31	0.31,0.45	0.03	0.11,0.31,0.45,0.5
3	130-150		0.88	0.88,0.91	
4	178	0.73, 0.89,0.41	0.41	0.58,0.61,0.75	0.61,0.75,0.89
5	216	0.61, 0.78	0.42,0.61,0.78	0.55,0.61,0.78	0.70
6	226-239		0.45	0.04,0.14,0.59,0.73,0.94	0.05,0.14,0.25

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5. l Discussion

Antimicrobial activity on the fractions of Petroleum ether & Dichloromethane was studied by measuring the zones of inhibition formed around the discs and the results were tabulated in Table 2 and represented graphically. Their potency was assigned with regards to inhibition zone diameter to such a degree that; Minimal antibacterial activity (< 7 mm), Active (8-11 mm), and Very active (> 12 mm) (Mwitari et al 2013).

Antimicrobial Assay of the Extract

Antimicrobial assay of the extract was carried out by agar well diffusion method in eight petri dishes. The test organisms (*Escherichia coli* & *Staphylococcus aureus*) were inoculated in nutrient broth & incubated overnight at 37^0 Celsius. The petri dishes were lawn cultured with standardized microbial culture broth. Six spots were marked in the inoculated media. For the first petri dish, the spots were filled with the fractions F1, F2, F178, F130-150; F216 &F226-239.This was done in duplicate for *E coli*. The same was done for *Staphylococcus aureus.* Specific quantities in mg included F1(136mg),F2(159mg),F130-150(240mg),F178(89mg),F216(123mg) & F229-239(143mg).Each quantity of the fraction was dissolved in 0.1% DMSO.Positive control was standard Ciprofloxacin 30mcg.The petri dishes were then incubated for 24hours at 37^0 Celsius. After incubation, the petri dishes were examined for the emergence of a distinguishable zone around the spot which corresponded to antibacterial activity of the tested fractions. The zone of inhibition was examined and measured in mm.

Thin Layer Chromatography was done on TLC plates and the plates were visualized by the normal eye, under short wavelength (254 nm) and long wave length (365 nm). The TLC plates were also visualized in the iodine chamber. Observed spots were circled normal eye

(E), short wavelength (S), long wave length (L) and iodine chamber (I).

5.2 Conclusion

Isolated fractions exhibited antibacterial activity. Fractions, F1 & F216 had maximum antibacterial activity (>12mm).F1 maximum activity was on *Staphylococcus aureus* whereas Fraction F216 maximum activity on *E coli*. The other four fractions F2, F130-150, F178 & F226-239 had minimal antibacterial activity. Fractions obtained from the root extracts of Uvariodendron anisatum can serve as a potent ingredient for herbal medicinal products.

5.3 Recommendation

There is need for further studies on bioassay guided fractionation and characterization of isolated fractions and later the studies on the efficacy as well as safety of this medicinal plant. Studies of *Uvanodendron anisatum* towards human cells should be carried out since there are no studies found in literature.

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APPENDICES

Figure 6: Escherichia coli

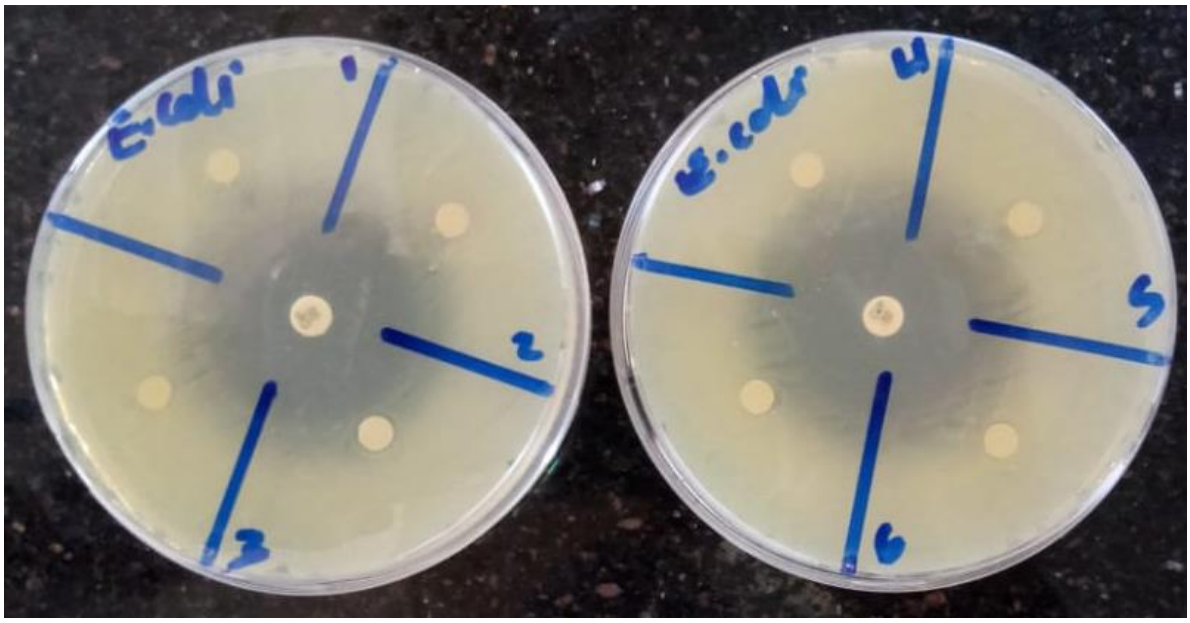

Figure 7: *Staphylococcus aureus*