ANTIMICROBIAL INVESTIGATIONS, PHYTOCHEMICAL CONSTITUENTS AND CYTOTOXICITY EVALUATION OF *Acokanthera schimperi* (A. DC.) Schweinf. LEAVES CRUDE EXTRACT

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(PHARMACOLOGY AND TOXICOLOGY)

DEPARTMENT OF PUBLIC HEALTH, PHARMACOLOGY AND TOXICOLOGY,

DECLARATION

I hereby declare that this is my original work and has not been presented before in this University or any other University for the award of this or any other degree.

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DEDICATION

First and foremost, The Almighty God with full observance to The Holy Trinity. Second, my late parents Dr. Zephaniah Kamau Mwangi and Jane Wangui Kamau, my two issues, Elianah Wangui Mwangi and Mireya Kemuma Mwangi. Lastly my loving wife Redempter for the moral support and helping me stay the course.

ISAIAH 60:22 …...When the time is right, I The Lord, will make it happen……...

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ACRONYMS AND ABBREVIATIONS

1.ATCC-------American Type Culture Collection

2.AMR---------Antimicrobial Resistance

3. PDAs--------Plant Derived Antimicrobials

4.TMP-----------Traditional Medicine Practitioner

5. WHO----------World Health Organization

6. PLWHIV--------People Living with Human Immunodeficiency Virus

7. NMR----------Nuclear Magnetic Resonance

8. MS----------Mass Spectrophotometry

9. HIV-AIDS--------Human Immunodeficiency Virus-Acquired Immunodeficiency Syndrome

10.1550 BC--------Year 1550 Before Christ

11.BLSA----------Brine Shrimp Lethality Assay

ABSTRACT

Phytomedicine, a significant area of study and an intervention towards alleviation of mankind and animal suffering using ethnomedicine and ethnoveterinary approaches, has catapulted bioprospection in drug discovery. This strategic approaches have led to our cynosure on *Acokanthera schimperi* (A. DC.) Schweinf. (Apocynaceae family). In the study, botanical identification and collection of plant voucher specimen was done in the company of both an herbalist and a Taxonomist. The latter was then deposited in the University of Nairobi Herbarium, School of Biological Sciences, Chiromo Campus where a voucher specimen number was issued. Approximately 5kg leaves of the study plant were sampled from several shrubs and transported to the Department of Pharmacology and Toxicology, University of Nairobi, College of Agriculture and Veterinary Services. Shade drying for two weeks was done after which, the leaves were pulverized using Chrisky Hunt machine 800 laboratory Mill at LARMAT to fine powder. About 4.5 grams of the dried powder was divided and put in three separate large conical flasks into which respective solvents were added. Hydroethanolic (50:50), distilled water and acetone (analytical grade) were the extraction solvents of choice. The conical flasks were air-tightly corked and put in a dark chamber for 24 hours to allow for extraction. Corresponding extracts obtained were labelled Hydroethanolic, Aqueous and Acetone extracts respectively. The determination of the extracts' cytotoxicity, antimicrobial activity and qualitative phytochemistry was done. The *in vitro* antimicrobial activity of the extracts were tested against standard reference organisms viz. *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus cereus* (ATCC 11778) and *Candida albicans* (ATCC 102231). Moreover, the safety of the generated extracts was evaluated using the BSLA using *Artemia salina* Leach. Qualitative phytochemistry analysis was also included in the study so as to identify secondary metabolites present in the extracts.

Analysis of laboratory data results by use of descriptive statistics involved two methods i.e. Kaber and One way Anova. In the analysis of variance, the acetonic leaf extract inhibited the growth of *Candida albicans* with a minimum inhibitory concentration value of 62.25 mg/ml, with a zone of inhibition of 29.5 mm. Comparatively, fluconazole as the positive control registered a zone of inhibition of 33 mm. The hydroethanolic extract inhibited the growth of *S. aureus, E. coli, P. aeruginosa* and *B. cereus* at concentrations between 250 mg/ml to 500 mg/ml. The aqueous extract inhibited the growth of *S. aureus, E. coli, P. aeruginosa* and *B. cereus* at concentrations between 125 mg/ml to 500 mg/ml. Both the aqueous and hydroethanolic *Acokanthera schimperi* extracts were non-cytotoxic to brine shrimp nauplii (LC_{50} 1000 µg/ml), and thus regarded to be safe. In contrast, the acetonic extract was moderately cytotoxic to brine shrimp nauplii (LC_{50} 451µg/ml), whereas vincristine with LC_{50} value of 289µg/ml was also moderately cytotoxic. Qualitative phytochemical screening showed that the three crude leaf extracts of *Acokanthera schimperi* (A. DC.) Schweinf. contained alkaloids, starch, glycosides, flavanoids, tannins, terpenoids, coumarins, volatile oils, anthraquinones and phenolic compounds. Cardiac glycosides were conspicuously absent. The presence of phenolic compounds and flavanoids could be the presumed antimicrobial pharmacological activity. Cytotoxicity studies implored that both the Aqueous and Ethanolic extracts were non-toxic at the dose concentrations of 10,100 and 1000 mg/ml hence registering 100% nauplii survival. However, the acetone extract at concentrations 100 and 1000 mg/ml executed all the 10 nauplii while none died at 10 mg/ml. In conclusion, *A. schimperi* is of pharmaceutical significance as the phytoconstituents present are indicative of a good template for drug discovery. The safety can be capped at weakly cytotoxic for the aqueous and hydroethanolic extracts. However, cardiac glycosides though absent in this study should be investigated in other studies as they are key in modern medicine. More studies are welcome to investigate the plant and more of its extracts for the betterment of healthcare.

CHAPTER ONE: INTRODUCTION

Nature through God's providence has offered relief in the availability of plants as a reservoir for medicine and supplements. Worldwide use of plants as medicine has increased exponentially with different populace using different medicinal plants for several ailments depending on their national healthcare setup.(Organization, 2004) (Barnes, 2003, Ekor 2014).

In developing countries, medicinal plants have been relied upon by at least 4 billion as a primary source of healthcare due to the inference of traditional medicine practice towards the provision of healthcare in present day (Bandaranayake, 2006) (Bodeker *et al.*, 2005)

Antimicrobial resistance being a global health concern has necessitated a one health approach where multi-displinary teams are charged with the responsibility of ensuring optimal health to plants, animals and the environment (McEwen and Collignon, 2018)

AMR has attracted a spirited fight towards the use of plant molecules. Phytochemicals though highly volatile have proved helpful. Plant derived antimicrobials (PDAs) when combined with convectional antibiotics give better and superior results in combating AMR.(Karthikeyan *et al.,* 2020). Due to the increase in microbes depicting resistance to population of different age cohorts worldwide, global networks of experts through advanced genomic and sequencing technologies have scaled up surveillance and outlined the need for urgent mitigation*(Abrudan et al.,* 2021). The integration of herbal therapeutic solutions as an adjunct to conventional medicine in 80% of world's population has proved synergistic. The secondary metabolites produced by the flora and fauna to date have been responsible for the pharmacological actions such as antidiarrheal, antisepsis, antifungal, antiacaricidal, anticancer, antimalarial and antiviral just to mention but a few(Kabera *et al.,* 2014)

Acokanthera schimperi (*Apocynaceae*) is a shrub native to Yemen, Ethiopia, Kenya, Somalia, Tanzania, S. Africa and Zimbabwe. It is found in well dried forests soils.

Notably, the belief that natural products are non-toxic and very safe is an outlier (Nasri, 2013). In fact accidental deaths have been witnessed due to insufficient knowledge of dosage prescription of medicinal plants (Farzaei *et al.*, 2020).

The plant is well known for cardiac glycosides that are mainly significant in hunting and warfare (Bisset, 1989). Most of the plant parts such as leaves, roots, stem, bark and whole plant have been used ethno medically courtesy of folklore (Omino and Kokwaro, 1993). Striking pharmacological activities include anti-inflammatory, anti-oxidant, anticancer, analgesic, antimicrobial etc. Moreover, its indicated in ailments such as headaches, rheumatic pain, warts, elephantiasis, scabies, eczema and common cold (Abebe and Ayehu, 1993), (Omino and Kokwaro, 1993).

In a study, antimicrobial activity carried out on crude methanolic leaf extracts of *A. schimperi* exhibited 100% growth inhibition of *S. pyogenes* while *Proteus vulgaris* and *Escherichia coli* were the most resistant (Taye *et al.,* 2011). However, in another study, *S. aureus* proved resistant to crude methanolic leaf extracts and these dynamics in activity could be attributed to change in plant season, solvent extraction techniques, or media used in screening (14). Furtherly, the essential oils from *A. schimperi* were studied and found to be averse to any antimicrobial activity as all the three microbes' namely; *C. albicans*, *E. coli* and *B. subtilis* registered growth with MICs greater than 128 mg/ml (Matebie *et al.,* 2019). In addition, a study involving the ethanolic leaf extract of *A schimperi* upon quantitative phytochemical screening proved to contain glycosides, tannins, Phenolics and steroids thus evidencing the antioxidant and free radical scavenging activity *(Chaithanya et al.,* 2020). Coumarins, being glycosides, present in *A schimperi* are of ethnoveterinary importance as they are known for their acaricidal property-tick control. (Owino *et al.*, 2015) .

The objective of the aforementioned research is to determine presence of secondary biomolecules by preliminary qualitative phytochemical analysis, cytotoxity by use of brine shrimp lethality assay and finally the antimicrobial activity as dictated by the phytoconstituents identified based on the acetonic, aqueous and hydroethanolic crude leaf extracts. It is therefore imperative to note that the use of plants as an infinite source of novel medicinal molecules is crucial to modern day human and veterinary medicine.

In so doing, the quest for more flora-derived medicines is on the rise with patients considering them safe and efficacious compared to modern day medicine. The antimicrobial agents, provided by natures biodiversity have led to compounds with tremendous combative power towards, bacteria, fungi, virus and protozoa with minute or no side effects(Gonelimali *et al.,* 2018). However, the study of natural products, plant extracts with their corresponding structures has led to exploration of novelties in research (Cowan, 1999). The art of mastery in practicing Ethnomedicine has led to accrual of knowledge and skill with databases established to boost scientific research on drug discovery(Pan *et al*., 2013).

Hope is rife in the discovery of novel Phytomolecules that can eradicate antimicrobial resistance (Multi-drug Resistant microbes) by the development of biocompatible remedies from folkloric knowledge (Anand *et al*., 2019). Regulatory bodies are key in the management and control of such discovery through preclinical and clinical trials hence avoiding poisoning and other fatalities (Farkas and Nattell, 2010).

A significant contribution of herbal medicine, for instance, is in reproductive health where women with fertility problems and also those without reach of modern fertility medicine have benefited.(James *et al*., 2018).This finding is dependent on the safety and effectiveness of herbal medicine which again is affected by the seasonality of the geographical niche area(Jamrozik and Selgelid, 2020) (Gololo *et al.,* 2016).

Acokanthera schimperi from the Dogbane family has been known not only to be a lethal plant especially during warfare due to its cardiac glycosides, but also rich in medicinal uses. However, the show of might by ethnic communities especially during border clashes has proved *Acokanthera schimperi* worthy(B. K. Cassels, 1985) .

1.2 Objectives

1.2.1. General Objective

To determine the *in vitro* antimicrobial activity, qualitative phytochemical screening and safety of crude extract from leaves of *Acokanthera schimperi.*

1.2.2 Specific Objectives

a. To evaluate the antimicrobial activity of organic and aqueous crude leaf extracts from *A. schimperi*
b. To analyze the cytotoxicity of organic and aqueous crude leaf extract of *A. schimperi* by using brine shrimp lethality assay (BLSA).
c. To characterize the phytochemical composition of crude extracts from the leaves of *A. schimperi*

1.3 Hypothesis

That the hydroethanolic, aqueous and acetonic crude leaf extracts from *A. schimperi* are rich in phytochemical constituents, possess potent *in vitro* antimicrobial activity on reference standard micro-organisms and are non-toxic to *Artemia salina* larvae.

1.4 Justification of Study

Ethnomedicine and ethno pharmacology has gained tremendous interest in present day as it has been known for therapeutic effects such as antidiarrheal, anticancer, antimicrobial, analgesic as well as other activities hence aiding in drug discovery (Süntar, 2020). The Maa community have integrated traditional medicine practice in their culture including sale of the same herbal medicines by TMPs. Numerous medicinal plants have exhibited ability to cure various diseases. Notably, scientific studies conducted only involve a fraction of the entire plant medicines (Oluyemisi *et al.,* 2012). A regulatory framework on proper use of such medicines is key to avoid a poisoned nation and audits ought to be done to identify posology gaps (Okumu *et al.,* 2017). On one hand, traditional medicine is seen as a breakthrough to medicine while on the other is disgraced as a poor man's remedy. The superiority or inferiority of such traditional medicine should be erased and seen as an alternative means of treatment together with other practices constituting medicinal practice (Patwardhan *et al.,* 2005). Secondary metabolites or Phytomolecules are constituents in medicinal plants aiding plants survival in the wilderness thus deterring their predation (Bennett and Wallsgrove, 1994). The presence of such phytomolecules has indeed narrowed the disease burden by the production of affordable, safe and efficacious medicines due to the pharmacological combativeness against pathogens. Folkloric data has shown *Acokanthera schimperi* as a very good source of both animal and human medicines. Evidenced data shows a plant remedy for nail infections, elephantiasis, tonsillitis, venereal diseases, headaches, skin fungal diseases and even wounds just to mention but a few. In animals the plant was used traditionally as an acaricidal agent, an insect and termite repellant by ethnic communities (Kenubih *et al*., 2021) (Alemu *et al.,* 2020) (Alemu *et al.,* 2020).

Here, we bring the solution to a ballooning ecosystem characterized by increasing disease burden to animals and humans but with keen interest to efficacious, safe and available plant

derived medicines. Our research intends to lower the burden of disease and give a limpid approach to health in a move to secure better livelihoods in both humans and livestock. These can only be purposed by production of socially acceptable, environmental friendly, safe and efficacious medicines.

CHAPTER TWO: LITERATURE REVIEW

The paradigm shift in medicine was first witnessed with the accidental discovery of Penicillin in the 19th century. Consequently, an explosive use of antibiotics was witnessed curbing both animal and human diseases. The irrational, and or abuse of such antibiotics has now led to AMR in the general population. The otherwise resistant bacteria and fungi have led to delay in treatment and thus universal suffrage courtesy of AMR (Jamrozik and Selgelid, 2020). Misdiagnosis, high disease burden, poor health infrastructure, poor control of any antimicrobial agent in markets against microbes are some of the factors that may lead to AMR. Moreover, in scenarios where 'superbugs' are involved, the choice of such biocides for therapy is limited (Das *et al*., 2017).

The emergence of novelties from flora and fauna is evident in the modern markets as scientists continue to rack their brains on new ethnomedicinal remedies. Overwhelming research of advantageous Phytomedicine that are dependable to the population in China is on the rise especially in this covid-19 era due to inadequate risk assessment (Gbadamosi, 2020).

To utilize and share such limited knowledge and resources optimally, certain protocols have come up with the view to protect and govern the same. Nagoya, Cartagena and other treaties are some of the agreements that countries have formulated to promote research on the utilization of biological diversity (Beck., 2019). Ethnomedicine especially, has played a definitive role depending on the community in question. TMPs have been the adage consultants on the remedies to ailments. Modern day specialists have now taken over in the 21st century. Alternative medicine remains a force to reckon with as the integration of traditional and modern medicine has evolved to fruition. (Zhang, 2000). It is worthy to note, conservation of such medicinal plants together with their cultivation is important so as not to erode their benefits. In fact human economic activities such as deforestation have led to medicinal plants being

endangered for existence(Rajeswara Rao *et al.,* 2012) (Mir *et al*., 2021). Infrastructure as pertains to validation of traditional medicine is on the rise with databases established for continuous referencing and conservation(Mukherjee *et al*., 2018).

Conversation measures such as education programs, establishment of conservation bodies, cultivation, database establishment, international treaties and any other efforts concerning the thriving of herbal medicines should be checked so as not to compromise quality and quantity of phytoconstituents. Such secondary metabolites if absent may affect the pharmacological aptitude of the said medicinal plant (SO *et al.,* 2018) . AMR's combative phenomenon has led to researchers acknowledging the use of medicinal plant remedies as cost effective. The synergy of medicinal plants and convectional antimicrobials with special attention to drug-drug interactions has been reported. (Stermitz *et al.*,2000). Multi-resistant TB being the apparent hazard has shown how weak antimicrobials are and hence the need for new and safe ones urgently. In fact, the replacement of outdated antimicrobial agents or even using them in a rotational manner could be a step in the right direction as far as AMR eradication is concerned. (Quale and Atwood.,1996)

2.1 Recognition of Traditional Medicine in Kenyan Constitution.

The Kenyan constitution provides a framework to address the health and safety threats that may affect its sovereignty and national development during governance technologies on ethnomedicine.(Harrington, 2018)

In addition, the constitution outlines Traditional Medicine (TM) as the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness (Tesfaye Gobena., 2013)

Ethnomedicine is defined as any knowledge, skill or practice beneficial in the diagnosis, prevention and curing disease based on bioactive from plants and animals used by various ethnic communities traditionally. In fact, WHO recognizes ethnomedicine as a therapeutic practice still in use in modern day without documentation on safety (Bishop., 2015). Traditional Medical Practitioners (TMPs) are defined as individuals known to the ethnic community as experts in health provision by use of flora and fauna while also employing indigenous spiritual, supernatural or even mystic service (Gakuya *et al.,* 2020)

Herbal formulations in ethnomedicine include boiled products, tinctures, concoctions, infusions, macerates and decoctions. The constituent phytochemicals are responsible for therapeutic effects present. Treatment of cancer, malaria, HIV-AIDS, fungi are some of the diseases that plant molecules have arrested.

Paclitaxel from *Taxus brevifolia*, vinblastine and vincristine (*Catharanthus roseus*), artemisinin from *Artemisia annua*, terbinafine from *Streptomyces species*, morphine from *Papaver somniferum* (analgesic), atropine (anticholinergic) from *Atropa belladona* just to mention but a few are some the ancient (Jin-Ming *et al.,* 2003).

2.2 Traditional and Herbal Remedies in Kenya

Therapeutic practices of traditional medicine have also deliberately been scaled up by cultural communities in Kenya. In Kisii county the prescription of antiphytoviral plants to improve the livelihoods of PLWHIV is documented.(Nyamoita *et al.,* 2020). Humans and animals have benefited immensely in treatment as the cost of conventional medicine increases. Ethnomedical surveys have supported the documentation of traditional medicine in Kenya (Nanyingi *et al.,* 2008).

The Table 2.1 below illustrates some communities with corresponding medicinal plants used in treatment of certain ailments in Kenya.

Table 2.1: Table below shows Medicinal plants used by different communities in treatment of disease

Plant Variety & Local Name	Family	Medicinal Use	Plant Part	Community	Reference
Acokanthera schimperi (Lamorijoi)	*Apocynaceae*	Acaricidal (anti-tick)	Leaves	Samburu	(Owino *et al.*, 2015)
Euclea divinorum Hiern (Usuet)	*Ebenaceae*	Toothbrush	Roots and Bark	Nandi	*(Pascaline et al*., 2011)
Combretum collinum Fresen (Buukwet)	*Combretaceae*	Infertility in women	Bark	Tugen	(Kigen *et al.*, 2016)
Agave sisilana (Makonket)	*Agavaceae*	Burns	Leaves	Sabaots	(Okello *et al.*, 2010)
Strychnos henningsii (Muteta)	*Loganiaceae*	Joint pains	Leaves	Kamba	(Gachathi and Hayashi, 1998)
Olea capensis	*Oleaeceae*	Ulcers	Leaves	Luhya	(Mukungu *et al*., 2016)
Carrisa edulis (Ochuoga)	*Apocynaceae*	Common cold, pneumonia and asthma	Roots	Luo	(Leonard *et al.,* 2016)
Acacia horrida(L) wild (Lerai)	*Mimosaceous*	Diarrhea	Bark	Samburu	(Omwenga *et al.,* 2014)
Urtica masaica (Hatha)	*Urticaceae*	Arthritis, Gout	Roots, Leaves	Kikuyu	(Kamau *et al.,* 2016)
Uraria acuminate Oliv.	*Annonaceae*	Drowsiness	Roots, Leaves	Mijikenda	(Muniafu *et al.,* 2014)
Asystasia mysorensis (Roth)	A*canthaceae*	Colds, headache and malaria	Leaves	Pokot	(Mbuni *et al*., 2020)

2.3 Medicinal Plants and Drug Discovery

Natural ligands derived from plant sources for medicinal purpose is a practice from time immemorial. Poppy, henbane and mandrake are some of the alkaloids from approximately 250 plants that were the earliest evidence of medicinal plant usage for preparations of drugs (De Pasquale, 1984). Again, in 1550 BC plant derived pomegranate, aloe, senna, fig, coriander, juniper, castor oil, willow and common centaury were used as therapy evidenced through prescriptions (Bhadra, 2020)

In the United States, The National Cancer Institute has redirected funding meant for drug discovery from medicinal plants. Focus has shifted to improving diagnosis and prevention together with the hastening of lead compounds from the development phase to clinical trials to reduce time lag in drug discovery(Gelmon *et al.,* 1999).

However, the time consuming analogy in drug development requires improvements in advanced technologies such as high-throughput screening and combinations in chemistry in drug discovery thus shortening the period.(Koehn and Carter, 2009)

Challenges in bioassay screening need to be resolved (Balunas and Kinghorn, 2005a) by using compound and extract libraries with new techniques that include prefractionation of such extracts thus reducing time of discovery(Bindseil *et al.,* 2001).

Modernization of the already existing NMR and MS methods of compound identification should be effected to facilitate faster and easier compound isolation (Balunas and Kinghorn, 2005a). The incorporation of high-throughput X-Ray crystallography could improve medicinal plant hit to lead drug discovery(Blundell and Patel, 2004).

Due to the complexity of structure of plant derived medicines and also low quantity during synthesis, collaboration between synthetic, medicinal chemists and pharmacognosists is required to advice on synthesis and semi-synthesis (Rout *et al*., 2009)

Nonetheless, it should be noted, even with the challenges, plants remain the best option in drug discovery and with innovative ways, quality and quantity can be enhanced(Jachak and Saklani, 2007).

In the recent ancient past (Morphine from *Papaver somniferum*), Galanthamine from *Galanthus waronowii*, Nitisinone from *Callistemon citrinus* and Triotropium from *Atropa belladonna* are some of the medicine derived from medicinal plants (Ateş and Yalçın, 2022). The compound vinflunine is an anticancer improvement of vinblastine and has good prognosis

in cancer patients (Balunas and Kinghorn., 2005b). In this regard various pharmacopoeia such as the British, United States and European have been established to standardize the grade of purity of such derived medicines for proper manufacturing and large scale. Anglo-Saxon pharmacopoeia has been isolated as of great advantage to isolation and identification of natural compound synthesis(Watkins *et al*., 2011). Notably, antagonism and synergy in natural products has been discussed as an interaction phenomenon among extracts, bearing in mind the complex matrix that helps in mechanism of action(K. Caesar and B. Cech., 2019).

Bioassays, phytochemical screening, journals, biological activity reports etc. are some of the avenues taken to identify potential lead compounds that are ready for lead optimization (Fabricant and Farnsworth., 2001).

The use of guinea pigs, mice, chicken, chimpanzees, monkey and rats for experimental studies should now be synergized using proper preclinical and clinical studies where alternative subjects such as human cells, tissues, organ models are involved for confirmation of safety and efficacious medicines. This synergy is key in hastening the medicines approval by the regulatory bodies. (Van., 2019)

2.4 Challenges Present in Drug Discovery from Medicinal Plants

Drug discovery through medicinal plants has been a true success. However, scientists need to keep up with the growing competition from synthetic drug discovery. Quantity and quality needs to be enhanced for this reason(Li *et al.*, 2020).

According to (Graz *et al.,* 2007), clinical trials from Phytomedicine can be conducted at a cost effective manner. Fundamentally, for proper drug discovery process, 10 years or more are required. Cost implications of more than 800 million dollars are pegged into such discoveries(Kalyaanamoorthy and Chen, 2011).

The process of medicine discovery involves lead identification, lead optimization, lead development, and clinical trials. These processes are time consuming and it is estimated that only one compound in 5000 gets to proceed from start to finish.(Subramanian, 2014). It is for this reason that drug companies have opted to scale down medicine production from plant origin thus affecting Phytomedicine research (Koehn and Carter, 2004).

2.5 Classification of Natural Products

The classification of flora and fauna based products according to, 'The origin and the nature of natural products by Cock 2008 employs four categories;

2.5.1 Classification by physiological activity

The active ingredient of a particular product may cause physiologically active effects from plant or animal origin. Natural products such as atropine produce mydriasis as a physiological activity.

2.5.2 Classification by chemotaxonomy

Here chemotaxonomy classifies phytoconstituents according to constituents in plants taxa. Constituents in plants are regarded as evolution makers in classification of plants.

2.5.3 Classification by molecular skeletal structure

Aromatic, benzoic, alicyclic, open-chain aliphatic and heterocyclic compounds are some of the key classification models thus natural products may be classified in this way.

2.5.4 Classification by biogenesis

Biosynthetic signaling pathways in the production of secondary metabolites can be used to classify natural products. Photosynthesis is the primary pathway in the synthesis of starch in plants by using carbon dioxide. Amino acids, lipids and nucleic acids also have synthetic pathways.

Acetate and amino acid pathways are responsible for formation of fatty acids, terpenes, polypeptides and alkaloids respectively. The building blocks of most phytoconstituents are part of these biosynthetic pathways and are derived from intermediates of acetyl coenzymes A, shikimic acid and mevalonic acid(Ikan ., 2007).

2.6 Literature on *Acokanthera schimperi*

2.6.1 The *Acokanthera* species (Apocynaceae).

The distribution of *Acokanthera* species is characteristic of rocky, wooded hillsides, kloofs, riverines, coastal bushes, open woodlands, scrub forests and woody areas(Botha and Venter, 2002).

Varieties include; *Acokanthera derflersii, Acokanthera friesiorum*, ***Acokanthera schimperi***, *Acokanthera spectabilis, Acokanthera longiflora, Acokanthera venenata* and *Acokanthera rhodesica* (Kupicha, 1982).

Botanically; these are evergreen shrubs which are of 3m in height but can reach up to 5m with rough-brown barks. The leaves are glossy, dark green on surface and paler below with a sharp, stiff summit. Leaves are also hairless with seldom red or purple trace colors. Leaf stalks are reddish with thick sweet scent and 10mm in length. Fruits are round to oval berries with colour changes from green (unripe), bright red and dark purple (ripe) (Botha and Venter, 2002). Figure 2.1 illustrates *A. schimperi* in the wild.

Figure 2.1: picture of *Acokanthera schimperi* courtesy of Kamau Joe Mwangi

2.6.2 Ecological niche of *Acokanthera schimperi* in Kenya.

Acokanthera schimperi is a shrub native to the Kenyan habitat and can be found in Narok, Mombasa, Samburu, Nandi and West Pokot. It grows in dry forest margins, grasslands and bush lands at 1100-2400 m attitude. The rainfall in this areas is 600-1000mm thus supporting growth. *Acokanthera schimperi* can be classified as a xeric shrub and prefers well drained black and red soils. The shrub is also cultivated by man (Maundu and Tengnas., 2005)

2.6.3 Uses of *Acokanthera schimperi*

Wood and roots of the shrub are used in the manufacture of arrow poison due to cardiac glycosides namely; Oleandrin, acavenoside A and ouabain. The preparation of such arrow poison is a result of boiling of the roots of *A. schimperi* and concentrate applied on the arrow heads (Bruce K. Cassels, 1985)

The unripe fruits are highly poisonous but when ripe are used as jam and eaten during drought/dry season. Toxicity of *Acokanthera schimperi* is seasonal throughout the year and is characterized by dead insects and birds around the tree area(A, 2008). In Samburu, unchecked menstrual flow is treated using a decoction from the barks of *A. schimperi*. The leaves are used to make decoction for the treatment of syphilis. Moreover, the plant is an aphrodisiac and used by the Kalenjin community (Jeruto *et al*., 2008). A unique phenomenon in toxicology of *A. schimperi* is that when roots and barks are masticated by the African crested rat, *Lophiomys imhausi,* the rodent smudges the masticates on its fur. Predators upon preying on the rodent die instantly as the poison is translocated in the fur of prey. Cardenolides present in the poisoned fur presumably cause death due to cardiotoxicity. This can be a very good avenue for study in drug discovery(Kingdon *et al.,* 2012).

In the pastoralist communities the leaf extracts have also been used for tick control where the hot decoction is applied cuteneously on cattle to control ticks (Owino *et al.,* 2015). In diverse cultures, especially in Ethiopia, *Acokanthera schimperi* is revered for its use in curative nature on wounds, jaundice, scabies, tonsillitis and rheumatic pain. *(Alemu et al.,* 2020)

2.6.4 Chemical composition of *Acokanthera schimperi* and their significance in modern medicine

Cardiotonics are associated constituents of *Acokanthera schimperi* variety, where ouabain is an example. The unripe fruits of the shrub are highly poisonous and depending on seasonality, toxicity is associated with the whole plant*(*Mijatovic *et al.,* 2007). Studies such as drug repurposing have shown the cardiac glycosides as stimulants of the heart rather than the usual cardiotoxic cardiotonic ingredients (Škubník *et al.,* 2021).

The notorious cardiac glycosides have been explored for antiviral activity thereby inhibiting every step in virion cycle except the attachment to the whole cell courtesy of drug repurposing

that has been brought about by the covid-19 era(Škubník *et al*., 2021). Interestingly, these has led to serious studies in drug repurposing and hence led to treatment of gastrointestinal stromal tumors thus harnessing anticancer properties of cardiotonics (Pessetto *et al.*, 2013). The main plant family sources of cardiac glycosides include, Asclepiadaceous, Apocynaceae, Moreaceae, Leguminasae, Tiliaceae, Celastraceae, Scrophulariaceae, Ranunculaceae and Cruciferae (Hollman, 1985).

The cardiotonic compounds in *A. schimperi* known include; Acavenoside A, with the aglycone acovenosigenin, Ouabain, Oendrin and traces of acovenosigenin A. Shrubs of *Acokanthera schimperi* growing in Nairobi have highest Acavenoside A and lowest ouabain levels. Those in the coastal region have high ouabain while those in Eritrea have just half as much acavenoside A. Geographical distribution as a factor of phytochemical presence is evident. Coumarin derivatives are also present in *Acokanthera schimperi* and include; 8-hydroxy-2H Chromen-2-one and (E)-methyl-4-hydroxy-7-oxo-5 (2-oxo-2h-chromen-8-glexy) 0ct-2-enoate. The two derivatives are active against, *Rhipicephalus appendiculatus* larvae, registering LC_{50} and LC_{90} of 2.96 mg/ml and 6.09 mg/ml in that order. This is an area of study requiring attention especially in formulation of acaricidal agents. (Owino *et al.,* 2015).

2.7 The significance of Medicinal Plants in research

2.7.1 Antimicrobial Activity

In Kenya, antimicrobial activity due to potent phytoextracts of *Olea Africana* stem-bark, *Psidium guajava*, *Lantana camara, Vernonia amygdalina* and *L. mangifera* leaves were tested against *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*. All extracts registered activity against *S. aureus* while *V. amygdalina* and *L. camara* lacked efficacy against *P. aeruginosa* and *E. coli. Olea Africana* and *P. guajava* exhibited lowest MIC against *S. aureus*. (Cheruiyot *et al.,* 2009)

In addition, a study involving chloroform, ethanol and water extracts of *Solanum aculeastrum, Erythrina abyssinica, Carrisa edulis, Croton megalocarpus* and *Myrica salicifolia* were tested against *Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa* and *Klebseilla pneumonia*. All water extracts showed highest antibacterial activity followed by ethanol and chloroform extracts respectively(Kariuki *et al*., 2014).

The methanolic and aqueous extracts of *Ajuga remota, Piliostigma thonningii, Ocimum suave, Erythrina abyssinica* and *Harissonia abyssinica* were investigated for their antibacterial activity against *Staphylococcus aureus, Bacillus aureus, Pseudomonas aeruginosa* and *Escherichia coli*. The most susceptible bacteria were *S. aureus* then followed by *B. cereus* while the most resistant was *P. aeruginosa. Methanolic extracts of P. thonningii stem* and *Ocimum suave* leaves had the best antibacterial activity among all the four bacterial species (Musau, 2011). It is therefore prudent to note that medicinal plants are an infinite source to drug discovery.

2.7.2 Antimalarial activity

The malaria burden in Africa as compared to the world makes it a public/international health menace. Poor response to chemotherapy, poorly controlled vectors and improper hygiene are factors promoting its thrive. Plants such as *Monodora angolensis, Strychnos usambavensis, Fagara zanthoxyloides* and others have been documented as medicinal plants that are effective against malaria with potent antiplasmodial activity. This may be due to the presence of alkaloid and terpenoid phytoconstituents(Amoa Onguéné *et al.,* 2013).

2.7.3 Trypanocidal activity

In a study of 30 medicinal plants, their trypanocidal and cytotoxic effects were studied. *Acokanthera schimperi, Ocimum urticifolium, Albiza schimperiana, Daryalis abyssiuca and Chenopodium ambrosioides* showed marked and selective biocidal activity against

trypanosomes i.e. *Trypanosoma brucei brucei* and also HL-60 (Human leukemia-60 cells) (Nibret and Wink, 2011).

Acokanthera schimperi leaf extract manifested its activity towards *Trypanosoma congolense*, a causative agent for sleeping sickness (Assefa, 2017).

Table 2.2: Medicinal plants depicting anticancer activity with phytochemicals responsible

Plant species	Plant part used	Model	Cytotoxic features	Compounds identified	Reference
Guazuma ulmifolia	Stem bark	K562 cells and mouse 657b1/6	Protected against doxorubicin induced cardiotoxicity and reduced invitro oxidative hemolysis	Citric & quinic acid	(dos Santos *et al.*, 2018)
Schinus terebinthifolia	Leaves	K562 cells and mouse 657b1/6	Protected against doxorubicin induced cytotoxity and used oxidative haemolysis	Phenolics, flavonoids tannin & ascorbic acid, α-pinene, limonene, cavene & phellandrene	(de Giffoni de Carballo *et al.*, 2019)
Harcornia speciosa	Leaves	Kasumi -1 cells	Necroptosis and cathepsin release	Kaempferol and rutin derived flavanoids, quinic acid Bornesitol and chlorogenic acid	(Santos *et al.*, 2016)
Campomanesia adamantium	Leaves	PC-3	Inhibited prostate cancer cell proliferation, DNA fragmentation and used NFKB, expression	B- mycerene, spathulenol, B-caryophyllene oxide, limonene & viridifloral	(Pascoal *et al*., 2014)
Jacaranda decurrens	Leaves	K562 cells	Mitochondrial depolarization, caspase -3 activation, necrosis & late apoptosis	Phenolic, compounds & flavonoids	(Casagrande *et al.,* 2014)

Surprisingly, the antimalarial compound artemisinin from *Artemisia annua* also evinced significant antitrypanosomal activity with an LC_{50} value of 35.9 mg/ml (Nibret and Wink,

2010). These therefore displays the use of medicinal plants in the prevention and cure of zoonotic diseases thus keeping humans and animals healthy.

2.7.4 Anticancer activity.

Anticancer exhibiting medicinal plants have shown activity by promoting longevity. The patient by simply remaining in remission indicates the importance of medicinal plants as potent anticancer agents. The table 2.2 above illustrates plant species with their inherent mechanism of action and compounds responsible for anticancer activity.

2.8 Extraction Methods for Phytochemicals Constituents.

Azwanida NN (2015) has published an article, 'A review on the extraction methods used in medicinal plants, principle, strength and limitation'. The article identifies the need for proper extraction of phytochemicals to maintain their quality and quantity. He also reported that the extraction methods though standardized, may vary from species to species depending on plant and disease treated. The extraction methods are vital but require more automation to avoid adulteration.

Extraction methods such as; Maceration, infusion, percolation and decoction are among the many methods in use. Other methods are also discussed.

- **Infusions or hot teas** are methods used for delicate herbs, fresh tender plants or leaves. The methods involve putting the material in a covered container with water and then boiled for 10-15 minutes. Sieving follows and bouillon is ready for administration (Organization *et al*., 2008).
- **Decoctions** are made from heat stable and harder materials like bark or roots. The plant parts are boiled for 20 minutes and strained, ready for administration. For stronger decoctions the bouillon is boiled for 2 hours or longer (Jaylor., 2004).

- **Maceration method** involves soaking the plant material overnight. This is done to avoid degradation of active ingredients by heat or alcohol. Cataplasms/poultices are prepared by crushing the herb by use of pestle and mortar spread on a piece of cloth and placed on the sick wound area
- **In percolation**, dried powders are packed in an equipment called percolator, containing boiling water and macerated for 2 hours. Percolation is done at moderate rate (6 drops/minute) until extraction is completed so as to concentrate the extracts.
- **Soxhlet extraction/hot continuous extraction** involves placing finely ground sample or herb in porous bag made of strong filter paper. The thimble chamber of Soxhlet apparatus then holds the porous bag. Menstruum (solvent for extraction) is heated at the bottom of a flask vaporizing in the porous bag. Condensation takes place and drips back to the flaks and process goes on.
- **Microwave Assisted Extraction (MAE);** In this extraction method, the use of microwave radiation in separation of analytes is key. Microwave energy interacts with dipoles of polar solvents. The solvent matrix is made up of molecules with dipoles that rotate due to electromagnetic radiation thus disrupting hydrogen bonding. This enhances migration of dissolved ions promoting sample penetration in matrix. For non-polar solvents, heat transfer is by dielectric absorption only as compared to heat transferred by conduction in polar solvents. MAE can be classified as a selective method that favors polar molecules and solvents with high dielectric constants.
- **Accelerated solvent extraction (ASE);** ASE is a more superior method of extraction compared to maceration and Soxhlet. The sample is packed in the stainless steel extraction cell with inert material such as sand. The inert material prevents sample from clogging the system tubes. The packed extraction cell includes layers of sand-sample mixture sandwiched between cellulose filter paper and sand layers. Extraction is timely

as it takes only an hour because it is automated and able to control temperature and pressure on its own. Solvent types are also critical in extraction as phytochemicals extracted are also solvent specific.

- **Ultrasound assisted extraction (UAE) or Sonication extraction;** Here, ultrasound raging from $20Kh_z$ to $2000Kh_z$ is used. Acoustic cavitation mechanical effect increases surface contact between samples and solvents and also permeability of cells walls. Physical and chemical properties of samples subjected to ultrasound are disrupted thus breaking plant cell wall. This helps release compounds and mass transportation of solvents into plant cell. The procedure can be used in both small and large-scale phytochemicals extraction as its simple and of low cost.
- **Supercritical fluid extraction (SFE);** For the extraction to take place a dense gas substance showing both gas and liquid physical properties at critical point is used. Pressure and temperature are key parameters in achieving critical point of substance. Here, the supercritical fluid (SCF) behaves more like a gas but has solvating properties of a liquid. For instance, carbon dioxide (CO_2) becomes a supercritical fluid at above 31.1^oC and 7380kPa and is preferred in extraction due to its excellent non-polar solvent properties, low cost and low toxicity. Because of its poor solubility, SC-CO_2 can be induced to extract polar solvents by addition of ethanol and methanol. SC-CO_2 produces analytes of concentrated forms as CO_2 evaporates at suitable temperatures. Supercritical solvents can easily be altered in strength by manipulation of temperature, pressure or by adding modifiers that reduce time of extraction. However, a major disadvantage is that it's very expensive initially(Azwanida., 2015).

CHAPTER THREE: MATEIALS AND METHODS

3.1 Plant Source Area.

Leaves of *Acokanthera schimperi* were obtained from Narok North Sub- County. The co-ordinates of specific location are the Kenya National Library Narok Branch of Longitudes-DMS 35 52'5.22" E and latitude-DMS 1 5'52.29" S.

The dry climate temperature in Narok ranges from 50 degrees F and 80 degrees F and has an elevation of 1862 meters or (6108.92 feet). Narok is also located 107.5km West North West of Nairobi Kenya. The average rainfall in Narok is averagely 775 millimeters of rainfall annually (climatestotravel.com. climate/Kenya/Narok).

According to the 2019 census by bureau of statistics, the population in Narok county is that of 1,130,703 people with livestock and sugar production as the main economic activity. *Acokanthera schimperi* is naturally occurring in Narok county, Narok-North Sub county near the National Narok library. The coordinates for the location include, the Kenya National Library Service Narok branch, NRK-BMT Road.

Narok county covers a surface area of 17, 921 km squared and is ranked 3rd largest county in Kenya. It has a population of 1,130,703. The county has six major constituencies i.e. Narok north, Narok east, Narok west, Narok south, Kilgoris and Emurua Dikirr. The life expectancy in Narok is below 61 years with a poverty rate of 23%. Narok county is designated number 33 out of 47 counties in Kenya. Economic activities include; farming, manufacturing (leather), pastoralism and mining (gold mining in Kilgoris).

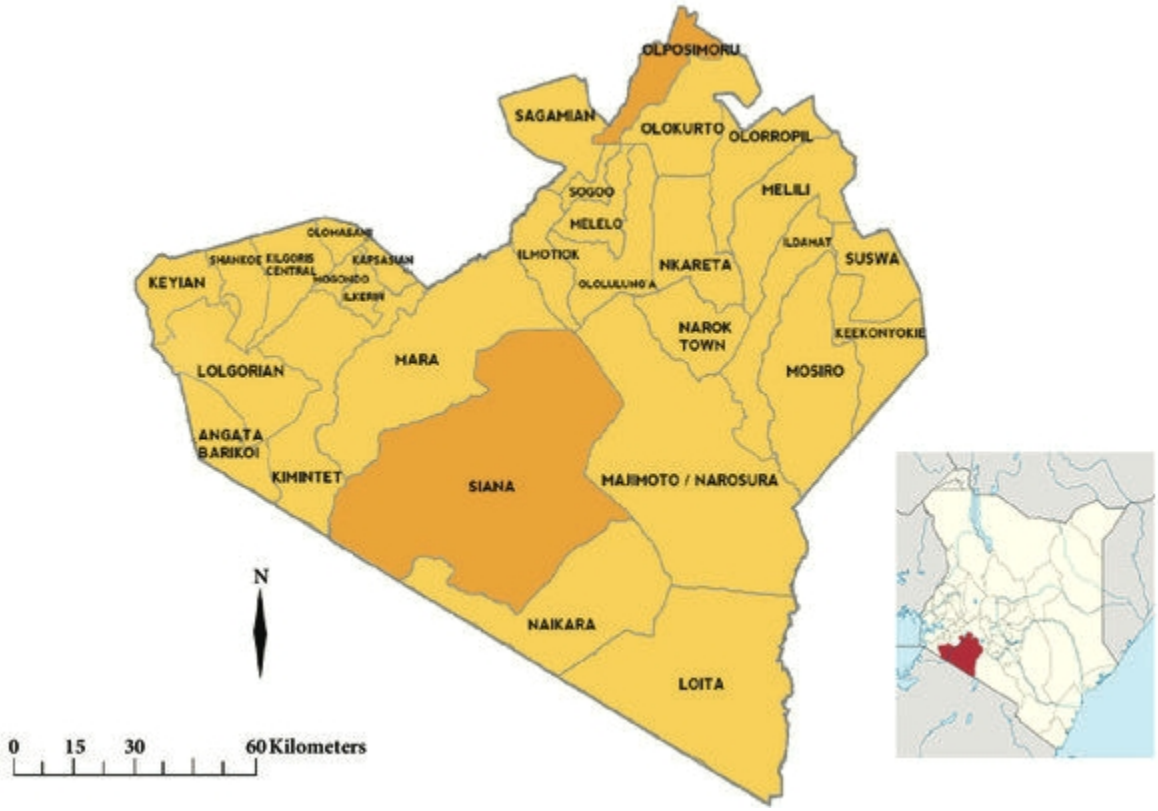

Figure 3. 1: Geographical map of Narok county.

In terms of health care provision, Narok county ranks numbers 16 out of 47 counties and approximately 90% of newborns are delivered in health centers by quality health personnel (KNBS, 2009).

3.2 Identification and Collection of *Acokanthera schimperi.*

An herbalist and a taxonomist are the two experts that identified and confirmed the actual variety of *Acokanthera schimperi* (see fig 3.2 below). Voucher specimen samples, three in number were obtained and preserved for delivery at the respective destinations. The samples were delivered at the University of Nairobi, Herbarium department- Biological sciences and a unique number of JMK UON 2021/001 given for the voucher samples. Approximately 5kg leaves were obtained for shade drying at the University of Nairobi, College of Agriculture and Veterinary Services-Lower Kabete campus. Information from the local herbalist and thorough

literature search made *A schimperi* significant to the Kenyan community and other communities in Africa.

Figure 3.2: Image of the team during *Acokanthera schimperi* collection. Behind is the plant under study

3.3 Preparation of Plant Samples for Laboratory Extraction

The leaves of *Acokanthera schimperi* were dried for a fortnight after which grinding followed using Chrisky Hunt machine 800 laboratory Mill at LARMAT. Pulverization was done in a fume chamber due to the dust powder emission. The powder was then packed in three large khaki bags and stapled ready for extraction.

3.4 Extraction Procedure in the UoN PHPT Lower Kabete Campus

About 1.5g of the powdered *Acokanthera schimperi* was put in each of three large conical flasks. Three litres of distilled water, hydroethanolic (50:50) and acetone (analytical grade)

were added into the conical flasks and extraction done through cold maceration. The conical flasks were stoppered and put in a dark chamber to soak for 48 hours. Shaking of the merc-menstrum mixture followed for two days to enhance percolation and extraction. The mixture was left to settle and filtration was done using Whatman filter paper (No. 1). The acetone extract was concentrated in a vacuum using a rotary evaporator. The resultant extract was further oven dried, after being transferred into a glass bottle, at 35ºc for 5 days.

In addition, hydroethanol and aqueous extracts were transferred into freeze-drying flasks, put into the freeze dryer and lyophilized for 48 hours. The extracts were weighed and percentage yields determined. After obtaining the extracts, storage at 40ºc was done awaiting further analysis.

3.5 Tests for Antimicrobial Activity

3.5.1 Tests for *in vitro* antimicrobial activity

3.5.1.1 Standard micro-organisms

The standard microorganism used for screening were *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778) and *Candida albicans* (ATCC 102231). Bacteria aforementioned were obtained from the Department of Public Health, Pharmacology and Toxicology, University of Nairobi. Disc diffusion and Broth microdilution methods described in the Clinical and Laboratory Standard Institute were at play.

3.5.1.2 Antimicrobial drugs used

Transchem pharmaceuticals was the source of Amoxyclav-625, a broad spectrum antibiotic and a positive control for gram-positive and gram-negative bacteria. Fluconazole 200mg (single tablet) was used as the positive control standard for antifungal activity.

3.5.1.3 Preparation and microbial inocula standardization.

The bacterial strains (*E. coli, S. aureus, B. cereus, P. aeruginosa and C. albicans*) were cultured in Mueller-Hilton and Sabourand dextrose Agar respectively as per CLSI guidelines for 24 hours. CLSI and not EUCAST guidelines were used because of the laboratory equipment and standard operating procedures present. The 0.5 McFarland scale of approximately $1\text{-}2\times10^8$ cfu/ml was used to standardize the inocula to an equivalent turbidity. Sterile normal saline was then used to standardize the inocula to achieve a 0.5 McFarland standard at 350nm by way of UV-Vis spectrophotometer. The OD530 value ranges of between 0.11 and 014 representing 1.5×10^6 cfu/ml, were obtained. The prepared standard microbes were used a stock cultures and kept refrigerated at +4ºc.

3.5.1.4 Investigation of antibacterial activity.

Using a sterile loop, a single colony was picked in each stock culture, introduced on pre-formed Mueller Hinton Agar (MHA) and incubated for 18 hours at 37ºc. Standard 1cm in diameter wells were punched on Agar plate.

The concentration gradients of positive control drugs used were 500 mg/ml, 250 mg/ml, 125mg/ml and 62.25 mg/ml.

For broth dilution technique, the inhibitory activity of plant extracts was used. Pre-sterilized Mueller Hinton Broth was dispensed into sterilized universal bottles and also in 10ml properly corked test-tube using sterile Pasteur pipettes. The tubes were put in a rack after labelling. Suspension of approximately 22×108 cfu/ml in sterile normal saline was prepared according to Shahidi 2004.

Stock solution of plant extracts of 500 mg/ml were prepared separately for each plant extract. From the stock solution two-fold serial dilutions were made in sterile Mueller Hinton Broth. 0.1 mm of bacterial suspensions was dispensed into the ten test-tube and incubated at 37ºc for

24 hours. Pure solvents were used as negative controls. The Minimum Inhibitory Concentration(MIC) and Minimum Fungicidal Concentration were defined as the lowest concentration that inhibited growth of bacteria/fungi on culture plate. Amoxyclav-625 and Fluconazole 200mg were used as standard antibiotics for positive control.

3.6 Test for Cytotoxicity Using Brine Shrimp Lethality Assay

3.6.1 Hatching of brine shrimp eggs

Into a 1 litre conical flask 33 grams of marine salt was weighed and transferred. Distilled water was then added gradually and stirred to homogeneity. Distilled water was topped up to the 1 litre mark to make marine salt solution.

In a shallow rectangular plastic double chambered box, brine shrimp eggs were hatched in 1.2 mm holes. The box was filled with the prepared marine salt solution. Using a spatula about 5mg of dry yeast was added after 50mg of brine shrimp eggs were introduced to the marine salt solution. The yeast (5mg) served as food for the nauplii in the dark compartment. The adjacent compartment was illuminated through a hole in the lid of the box using 40 watts' electric bulb. After 48 hours, the stimuli (light) attracted nauplii and Pasteur pipette used to collect them. Brine shrimp lethality test was then performed.

Figure 3.3: Monitoring process during hatching of brine shrimp eggs

3.6.2 Cytotoxicity bioassay technique

This bioassay was as per (McLaughlin., 1991). Three dilutions of extracts were prepared by transferring 500µl, 50µl and 5µl to an assay of five graduated test tubes. Ten shrimp nauplii were transferred into each of the vial using Pasteur pipettes and marine salt solution topped up to 5ml mark to produce a final concentration of 1000 µg/ml, 100 µg/ml and 10 µg/ml. Five graduated vials were set for each dilution and a further five for test control purposes. After 24 hrs. exposure, live nauplii were counted and LC_{50} calculated using Probit method of Finney

computer program. DMSO was used as negative control. In case of deaths in 24 hrs., data was corrected using formulae; % death=∑{(test control)/control)} ×100.

The Finney computer program provided by the Department of Pharmacology and Toxicology, Faculty of Agriculture and Veterinary Services used the numbers of dose level, number of shrimp for every concentration, percentage mortality for every concentration and dose level. The LC_{50} and 95% confidence intervals were determined using the computer program.

NB: ALL THE TESTS WERE DONE IN TRIPLICATE

Table 3.1: Different test procedures in qualitative phytoconstituents detection

Test	Procedure	Observations (indicating positive test)	References
Picric acid test (Alkaloids)	Few ml filtrate[a] +3-4 drops of 2% picric acid test	An orange colour	(Deshpande *et al.,* 2014) (Shanuvas and Indhumathi, 2018)
Keller-Killani test (Cardiac Glycosides)	1ml filtrate + 1.5 ml glacial acetic acid + 1 drop of 5% ferric chloride + conc. H_2SO_4 (along the side of test tube)	A blue colored solution (in acetic acid layer)	(Nanna *et al.*, 2013)& (Singh and Kumar, 2017)
Test for starch (Carbohydrates)	Aqueous extract +5ml 5% KOH solution	A canary coloration	(Audu *et al.,* 2007)
Borntrager's test (Glycosides)	2ml filtrated hydrolysate[c] + 3ml Chloroform + shaken well + chloroform layer separated + 10% Ammonia solution	A pink colored solution	(Raaman, 2006)
Ammonia test (Flavonoids)	Filtrate +5ml dil. Ammonia solution + conc. H_2SO_4	A yellow colour	(Ayoola *et al.*, 2008)
Braymer's test (Tannins)	1ml filtrate[d] + 3ml distilled water + 3 drops 10% Ferric chloride solution	Blue-green colour	(Singh and Kumar, 2017) (Ks *et al.,* 2017)
Terpenoids test (Terpenoids)	2ml chloroform + 5ml plant extract, (evaporated on water bath) +3ml conc. H_2SO_4 (boiled on water bath)	A grey colored solution	(Gul *et al.,* 2017)
NaOH paper test (Coumarins)	0.5 gm moistened extract is taken in test tube, mouth test tube is covered with IN NaOH treated filter paper, heated for a few min. in water bath	Yellow fluorescence from paper under the UV light	(Singh and Kumar, 2017)
Fluorescence test (Volatile oils)	10ml of extract, filtered till saturation, exposed to UV light	Bright pinkish fluorescence	(Mallhi *et al*., 2014)

Borntrager's test (Anthraquinones)	10ml 10% ammonia sol. + few ml filtrate[g] (Shaken vigorously for 30 sec.	A pink, violet, or red colored solution	(Njoku *et al*., 2011) (Ks *et al*., 2017)
Salkowski's test (Triterpenoids)	Filtrate[f] + few drops of conc. H_2SO_4 (shaken well and allowed to stand)	Golden yellow layer (at the bottom)	(Singh and Kumar, 2017)
Iodine test(Phenolic compounds)	1ml extract + few drops of dil. Iodine sol.	A transient red colour	(Singh and Kumar, 2017)

[a]=50gm solvent free extract is mixed with few ml dil. HCL and then filtered

[c]=50gm of plant extract is hydrolyzed with conc. HCl for 2hr on water bath and filtered

[f]=Equal quantity of chloroform is treated with plant extract and filtered

3.7 Phytochemical Screening

Aqueous, hydroethanolic and acetonic *Acokanthera schimperi* extracts were subjected to qualitative phytochemical screening in the identification of phytoconstituents using standard protocol. The following compounds were tested and Table 3.1 below summarizes the same;

3.8 Ethical Clearance

Approval to carry out research was sought from the University whereby an introductory letter was awarded. The researcher afterwards used the letter to seek research permit from NACOSTI and a research approval was issued. After acquiring all approvals, the researcher proceeded to collect research materials and carried out the tests as per study objectives.

CHAPTER FOUR: RESULTS

4.1 Significance of *Acokanthera schimperi*

In the course of our study the Narok community enumerated the various parts of the plant with which ailments are treated. In human health care the community uses *A. schimperi* leaves as a mosquito repellant and syphilis treatment. For gallbladder ailments treatment leaves and barks are the main parts used. In the event of uncontrolled menses, bark decoction is of choice. Ethnoveterinary importance of *A. schimperi* is evidenced by the control of ticks in livestock i.e. acaricidal properties.

4.2 Antimicrobial properties of *A. schimperi*

Results on the *in vitro* antimicrobial properties of the three plant extracts is shown in table 4.1,

Table 4.1: Therapeutic window and LC50 VALUES of tested crude extracts from *A. Schimperi*

Test Organisms/ Standard Microbes	Hydroethanolic Extract			Aqueous Extract			Acetone Extract		
	LC_{50} µg/ml	MIC	SI	LC_{50} µg/ml	MIC	SI	LC_{50} µg/ml	MIC	SI× 10^{-3}
Escherichia coli (ATCC 25922)	1000	125	8	1000	250	4	0451	250	1.804
Staphylococcus aureus (ATCC 25923)	1000	125	8	1000	62.25	16.064	0451	250	1.804
Pseudomonas aeruginosa (ATCC 27853)	1000	125	8	1000	62.25	16.064	0451	250	1.804
Bacillus cereus (ATCC 11778)	1000	500	2	1000	250	4	0451	125	3.608
Candida albicans (ATCC 102231)	1000	125	8	1000	250	4	0451	62.25	7.245

Table 4.2: Antimicrobial properties of *A. schimperi* crude extracts

Standard Organisms	CONC in mg/ml	MEAN ZONE OF INHIBITION (ZOI), MBC AND MFC CONCENTRATIONS of EXTRACTS									
		Hydroethanolic		Aqueous		Acetone		Positive Control		Negative Control	
		ZOI (mm)	MBC	ZOI(mm)	MBC	ZOI(mm)	MBC	ZOI(mm)	MBC	ZOI(mm)	MBC
Escherichia coli	500	28.83±0.24^{a}	250	25.33±0.58^{b}	250	15.33±0.76^{c}	250	30.83±1.77^{a}		0.00±0.00^{d}	
	250	27.50±0.50^{a}	0	24.00±1.00^{b}		14.00±1.00^{c}		29.33±1.41^{a}		0.00±0.00^{d}	
	125	0.00±0.00^{d}	0	0.00±0.00^{d}		0.00±0.00^{d}		28.50±0.35		0.00±0.00^{d}	
	62.5	0.00±0.00^{d}	0	0.00±0.00^{d}		0.00±0.00^{d}		26.00±1.41		0.00±0.00^{d}	
	31.25	0.00±0.00^{d}		0.00±0.00^{d}		0.00±0.00^{d}		24.50±0.71		0.00±0.00^{d}	
	15.625	0.00±0.00^{d}		0.00±0.00^{d}		0.00±0.00^{d}		23.83±0.35		0.00±0.00^{d}	
	7.8125	0.00±0.00^{d}		0.00±0.00^{d}		0.00±0.00^{d}		19.50±0.71		0.00±0.00^{d}	
	3.90625	0.00±0.00^{d}		0.00±0.00^{d}		0.00±0.00^{d}		11.17±1.06		0.00±0.00^{d}	
	1.953125	0.00±0.00^{d}		0.00±0.00^{d}		0.00±0.00^{d}		7.00±0.71		0.00±0.00^{d}	
Staphylococcus aureus	500	29.17±0.29^{a}	250	27.00±0.00^{a}	125	12.83±0.29^{d}	250	30.00±2.83^{a}		0.00±0.00^{e}	
	250	27.17±0.29^{a}		23.17±1.04^{b}		11.83±0.76^{d}		29.50±0.71^{a}		0.00±0.00^{e}	
	125	0.00±0.00^{e}		16.83±0.76^{d}		0.00±0.00^{e}		27.00±0.71		0.00±0.00^{e}	
	62.5	0.00±0.00^{e}		0.00±0.00^{e}		0.00±0.00^{e}		26.67±0.35		0.00±0.00^{e}	
	31.25	0.00±0.00^{e}		0.00±0.00^{e}		0.00±0.00^{e}		25.17±0.71		0.00±0.00^{e}	
	15.625	0.00±0.00^{e}		0.00±0.00^{e}		0.00±0.00^{e}		24.00±1.77		0.00±0.00^{e}	
	7.8125	0.00±0.00^{e}		0.00±0.00^{e}		0.00±0.00^{e}		18.83±1.06		0.00±0.00^{e}	
	3.90625	0.00±0.00^{e}		0.00±0.00^{e}		0.00±0.00^{e}		15.50±1.41		0.00±0.00^{e}	
	1.953125	0.00±0.00^{e}		0.00±0.00^{e}		0.00±0.00^{e}		12.67±0.71		0.00±0.00^{e}	
Pseudomonas aeruginosa	500	29.17±0.29^{a}		23.17±0.29^{b}		9.00±0.50^{d}		31.33±1.41^{a}		0.00±0.00^{e}	
	250	26.17±0.29^{a}	250	17.00±0.50^{c}	125	8.33±0.29^{d}	250	29.50±0.00^{a}		0.00±0.00^{e}	
	125	0.00±0.00^{e}		12.67±0.58^{c}		0.00±0.00^{e}		28.33±1.41		0.00±0.00^{e}	

	62.5	0.00±0.00 ^{e}		0.00±0.00 ^{e}		0.00±0.00 ^{e}		26.00±0.71	0.00±0.00 ^{e}
	31.25	0.00±0.00 ^{e}		0.00±0.00 ^{e}		0.00±0.00 ^{e}		25.17±1.06	0.00±0.00 ^{e}
	15.625	0.00±0.00 ^{e}		0.00±0.00 ^{e}		0.00±0.00 ^{e}		24.00±0.00	0.00±0.00 ^{e}
	7.8125	0.00±0.00 ^{e}		0.00±0.00 ^{e}		0.00±0.00 ^{e}		22.00±0.71	0.00±0.00 ^{e}
	3.90625	0.00±0.00 ^{e}		0.00±0.00 ^{e}		0.00±0.00 ^{e}		19.67±0.00	0.00±0.00 ^{e}
	1.953125	0.00±0.00 ^{e}		0.00±0.00 ^{e}		0.00±0.00 ^{e}		15.33±0.00	0.00±0.00 ^{e}
Bacillus cereus	500	15.50±0.50^{b}	500	13.50±0.50^{b}	250	15.17±1.26^{b}	250	29.00±0.35^{a}	0.00±0.00^{d}
	250	0.00±0.00^{d}		10.50±0.50^{c}		13.33±0.29^{b}		26.83±0.00^{a}	0.00±0.00 ^{d}
	125	0.00±0.00 ^{d}		0.00±0.00 ^{d}		0.00±0.00 ^{d}		25.00±0.71	0.00±0.00 ^{d}
	62.5	0.00±0.00 ^{d}		0.00±0.00 ^{d}		0.00±0.00 ^{d}		23.67±0.00	0.00±0.00 ^{d}
	31.25	0.00±0.00 ^{d}		0.00±0.00 ^{d}		0.00±0.00 ^{d}		21.83±0.35	0.00±0.00 ^{d}
	15.625	0.00±0.00 ^{d}		0.00±0.00 ^{d}		0.00±0.00 ^{d}		19.83±0.35	0.00±0.00 ^{d}
	7.8125	0.00±0.00 ^{d}		0.00±0.00 ^{d}		0.00±0.00 ^{d}		19.00±0.00	0.00±0.00 ^{d}
	3.90625	0.00±0.00 ^{d}		0.00±0.00 ^{d}		0.00±0.00 ^{d}		17.33±0.71	0.00±0.00 ^{d}
	1.953125	0.00±0.00 ^{d}		0.00±0.00 ^{d}		0.00±0.00 ^{d}		0.00±0.00	0.00±0.00 ^{d}
Candida albicans	500	9.17±0.29^{c}	500	7.33±0.58^{c}	250	29.50±0.50^{a}	62.25	33.00±0.71^{a}	0.00±0.00^{d}
	250	0.00±0.00^{d}		6.17±0.29^{c}		26.83±0.29^{b}		30.17±0.71^{a}	0.00±0.00 ^{d}
	125	0.00±0.00 ^{d}		0.00±0.00 ^{d}		24.00±0.00^{b}		28.83±0.35	0.00±0.00 ^{d}
	62.5	0.00±0.00 ^{d}		0.00±0.00 ^{d}		20.50±0.50^{b}		27.67±0.00	0.00±0.00 ^{d}
	31.25	0.00±0.00 ^{d}		0.00±0.00 ^{d}		0.00±0.00 ^{d}		26.00±0.71	0.00±0.00 ^{d}
	15.625	0.00±0.00 ^{d}		0.00±0.00 ^{d}		0.00±0.00 ^{d}		0.00±0.00	0.00±0.00 ^{d}
	7.8125	0.00±0.00 ^{d}		0.00±0.00 ^{d}		0.00±0.00 ^{d}		0.00±0.00	0.00±0.00 ^{d}
	3.90625	0.00±0.00 ^{d}		0.00±0.00 ^{d}		0.00±0.00 ^{d}		0.00±0.00	0.00±0.00 ^{d}
	1.953125	0.00±0.00 ^{d}		0.00±0.00 ^{d}		0.00±0.00 ^{d}		0.00±0.00	0.00±0.00 ^{d}

KEY: Zone of Inhibitions (ZOI) are the **mean ± standard deviation**; Positive controls : **[Amoxyclav-625mg And Fluconazole 200mg]**; Negative Controls: **[Micro Broth - Mcb / DMSO Or Water]**

Data was subjected to preliminary ANOVA to detect significant differences among means in a group and later subjected to post hoc ANOVA using Turkey test to separate means and compare tow means at ago to detect any significant differences between any of them. All the analysis was done is SPSS version 23 and the results have been summarized in Table 4.2. Means flagged with the same superscript letter within the considered group/row (e. g comparison of inhibition zones among various concentrations of hydroethanolic, aqueous and acetone extract against E. coli) are not significantly different ($p \geq 0.05$), while means flagged with different letters implies that they are significantly different ($p \leq 0.05$). For instance, in the comparison of hydroethanolic extract concentrations against E. coli, the positive control mean is flagged with 'a', 500 and 250 mg/ml flagged with superscript 'a' as well implying that inhibition at E. coli by 500 mg/ml and 250 mg/ml hydroethanolic extract of A. schimperi was not statistically significantly different from the inhibition of E.coli by the positive control at 500 and 250 mg/ml. On the contrary, the mean inhibition of E. coli by aqueous and acetone extracts at 500 and 250 mg/ml are flagged with 'b' and 'c' respectively while the other inhibition means from the other concentrations of A. schimperi against E. coli under the various extracts have been flagged with 'd' just like the negative control inhibitions which have been flagged with 'd'. This implies that the means flagged with 'b' are significantly different from the means flagged with 'c' which are also different from the negative control inhibition or the other means from the other concentrations flagged with 'd' at a probability level of 0.05.

In the broth dilution and Agar well diffusion, statistically significant antimicrobial activity was seen with the acetone, aqueous and hydroethanolic extracts. A remarkable antifungal activity was noted in the acetonic extract at an MIC of 62.25mg/ml. In agar well diffusion the ZoI of acetonic extract being 29.5mm exhibited a good fungal inhibitor to *C. albicans* compared to the positive control of 33mm for Fluconazole. The weak and/or no antibacterial activity on broth dilution between concentrations 250mg/ml and 500mg/ml for the Aqueous and Hydroethanolic extract was reported for *C. albicans*. At concentrations between 125mg/ml and 250mg/ml, tremendous antibacterial activity was seen in the hydroethanolic extract as inhibition of *S. aureus*(29.17mm)*, E. coli* (28.83mm)*, P. aeruginosa* (26.17mm) and *B. cereus* (15.50mm) respectively was vivid. There was very weak activity towards the *C. albicans.* However, the trend was similar in the aqueous extract with *S. aureus* (27.00mm), *E. coli* (25.33mm), *P. aeruginosa* (17.00mm) and *B. cereus* (13.50mm) with no significant activity in *C. albicans.* Notably, the hydroethanolic extract registered the highest antibacterial activity followed by the aqueous extract in the five standard organisms. Both extracts also evinced very weak antifungal activity.

4.3 Cytotoxic activity of *Acokanthera schimperi* on Brine shrimp larvae

Both the aqueous and hydroethanolic *Acokanthera schimperi* extracts were non-cytotoxic to brine shrimp nauplii (LC_{50} 1000 µg/ml) and thus regarded to be safe. In contrast, the acetonic extract was moderately cytotoxic to brine shrimp nauplii (LC_{50} 451µg/ml), while vincristine with LC_{50} value of 289µg/ml was moderately cytotoxic. Table 4.3 shows the cytotoxicity activity of *A. schimperi* extracts

Table 4.3: The effect of Aqueous, Hydroethanolic and Acetonic extract on Brine shrimp nauplii

BRINE SHRIMP LETHALITY TESTS SCORE SHEET.								
NUMBER OF DEAD NAUPILI IN EACH TUBE AFTER 24 HRS INCUBATION								
Extract ID	**Tube number**	**1**	**2**	**3**	**4**	**5**	**AVR**	**%Mortality**
	Concentrations							
Aqueous extract.	10^1	0	0	0	0	0	0	0
	10^2	0	0	0	0	0	0	0
	10^3	0	0	0	0	0	0	0
Ethanolic extract.	10^1	0	0	0	0	0	0	0
	10^2	0	0	0	0	0	0	0
	10^3	0	0	0	0	0	0	0
Acetonic extract.	10^1	0	0	0	0	0	0	0
	10^2	1	2	1	3	3	2	20
	10^3	10	10	10	10	10	10	100
Vincristine	10^1	2	1	2	0	0	1	10
	10^2	5	4	7	4	5	5	50
	10^3	10	10	10	10	10	10	100
1 % DMSO	10^1	-	-	-	-	-	-	-
	10^2	-	-	-	-	-	-	-
	10^3	0	0	0	0	0	0	0

4.4 Phytomolecules constituted in *Acokanthera schimperi*

Qualitative phytochemical screening revealed the presence of alkaloids, carbohydrates, glycosides, flavanoids, tannins, terpenoids, coumarins, volatile oils, and phenolic compounds. The presence of phenolics and flavanoids in *Acokanthera schimperi* leaves could be responsible for the observed amazing antimicrobial activity. Cardiac glycosides, a fingerprint constituent in *A. schimperi*, were absent in this case. The table 4.4 below is indicative of the secondary metabolites present.

Table 4.4 : Qualitative Phytochemical constituents of *Acokanthera schimperi*

	EXTRACTS		
Secondary Metabolites	**Acetone**	**Aqueous**	**Hydroethanol**
Alkaloids	-	++	+++
Cardiac Glycosides	-	-	-
Starch	-	+++	++
Glycosides	+++	++	+
Flavonoids	++	++	-
Tannins	+++	-	++
Terpenoids	-	++	+
Coumarins	+++	++	+
Volatile oils	+++	+	+
Anthraquinones	-	-	-
Triterpenoids	-	-	-
Phenolic Compounds	++	++	-

Key: +++ Higher concentration; ++ Moderately higher concentration; + Lower concentration; **-** Negative Results

CHAPTER FIVE: DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

5.1.1 Phytochemical constituents

Concentration of plant constituents varies with differences in soils, seasons, plant age, climate, plant geographical location and plant species of study. The Narok derived plant was found to contain; alkaloids, carbohydrates, glycosides, flavanoids, tannins, terpenoids, coumarins, volatile oils, and phenolic compounds hence its ethnoveterinary and ethnomedicinal significance. The antifungal activity of the plant extract against *C. albicans* could be due to phenolic compounds and tannins present as they are characteristic for the activity. The antimicrobial activity is well attributed terpenoids, flavanoids and alkaloids as seen with *S. aureus* and *E. coli*. Volatile oils present could be tested further for their significance. Cardiac glycosides though absent could be a significant area of study with sampling of the same plant done in another region to incorporate them as a constituent. Further screening of coumarins, anthraquinones and carbohydrates could be done to test their potential. As full development of

plant defense structures and systems continues, the concentration of phytomolecules decreases with increase in age of the medicinal plant. Due to the availability of the herb readily and at low cost could be the reason why the community opts to use the said herb rather than visit a health facility or call a veterinary officer. Though health aid from government and non-governmental agencies to livestock and humans at no cost is available, not all the communities benefit thus cultural practices in the use of the herb are slowly fading away.

5.1.2 Antimicrobial Activity

In vitro antimicrobial assays are important as they give justification on ethnomedicinal use of medicinal plants. The results of this study indicate a plant with broad-spectrum of antimicrobial activity as the three crude extracts show inhibition of *S. aureus, E. coli, P. aeruginosa, C. albicans* and *B. cereus.* The MIC values ranged from 125mg/ml and 500mg/ml for both hydroethanolic and aqueous leaf extracts. As for the acetonic extract MIC was 62.25mg/ml. The MBC values for the extracts also ranged from 62.25 to 500mg/ml. In this regard it is prudent to say *A. schimperi* has potent antimicrobial activity. The presence of secondary metabolites favoring antimicrobial activity such as tannins, phenolic compounds, flavanoids, alkaloids and triterpenoids could be the probable explanation for its remarkable activity. Narok community traditional herbalists' theory on use of the medicinal plant as remedy to wound healing in livestock and humans is somewhat justifiable.

5.1.3 Cytotoxic Assay by use of *Artemia salina* larvae

The safety of plant crude extracts can only be extrapolated by cytotoxic testing by several techniques.

Here, a dose dependent relationship to the death of nauplii was observed to some extent only with the acetone extract. Infact, no deaths of nauplii were recorded in the aqueous and hydroethanolic extracts thus weakly cytotoxic. At concentrations of 100 and 1000µg/ml a

mortality of 20 and 100% respectively was registered. *A. schimperi* acetonic extract registered LC50=451µg/ml which was comparable to that of vincristine LC50=289µg/ml. Both were classified as moderately cytotoxic.

Medicinal plant extract testing especially in bioassay of cytotoxicity using brine shrimps for lethality determination is a practice widely used. Medicinal plant extracts exhibiting no lethality to the nauplii do not necessarily mean that they are deficient of biological activity. In this study the two extracts, aqueous and hydroethanolic extracts showed no lethality but biological activity was present. This therefore justifies the use of *Acokanthera schimperi* as an herbal remedy to the Narok Community.

5.2 Conclusion

1. The aqueous, hydroethanolic and acetone extracts of *Acokanthera schimperi* manifested both antibacterial and antifungal activity against the standard organisms used. The hydroethanolic extract of *A. schimperi* exhibited significant antibacterial activity showing higher antibacterial activity against *S. aureus, E. coli, P. aeruginosa*, and *B. cereus* respectively than the aqueous extract. Aqueous extract also showed significant antibacterial activity in the same order of organism susceptibility as the hydroethanolic extract but with lower potency. Both the hydroethanolic and aqueous extracts showed weak or no antifungal activity
2. Antifungal activity of the acetone extract against *C. albicans* could be due to the non-polar nature of extraction acetone solvent
3. The aqueous and hydroethanolic extracts were found non-toxic to nauplii at concentrations of 1000µg/ml with 100% survival.

5.3 Recommendation

1. Where the scope concerns ethnobotany and laboratory screening of *A. schimperi*, further qualitative and quantitative phytochemical screening, antibacterial and toxicological studies need to be carried out so as to qualify and quantify other phytomolecules that maybe present depending on the plant's geographical origin.
2. Further *in vivo* laboratory profiling on toxicity and posology determination of *A. schimperi* products is required to understand the significance of the plant in treatment of patients.
3. Scientists involved in natural products analysis need to identify all active phytoconstituents and consider assessing all phototherapeutic products through clinical trials.
4. In the use of poisonous fur as a deterrence to predation by the "African Crested Rats," due to the smearing of *Acokanthera schimperi* masticates on its body, is an area that needs further study.
5. Prudence should be observed in training and posology to arrest malpractices in the administration and dispenses of *A. schimperi* products
6. In the bioprospection practice, narrowing into lead compounds that are specific to *A. schimperi* constituents should be done to curb side effects and adverse drug events that may cause harm to patients.

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APPENDICES

Appendix I: Ethical Clearance Certificate

UNIVERSITY OF NAIROBI
FACULTY OF VETERINARY MEDICINE
DEPARTMENT OF VETERINARY ANATOMY AND PHYSIOLOGY

P.O. Box 30197,
00100 Nairobi,
Kenya.

Tel: 4449004/4442014/ 6
Ext. 2300
Direct Line. 4448648

REF: FVM BAUEC/2022/337

Kamau Joe Mwangi.
Dept. of PHP & Toxicology
University of Nairobi
01/01/2022

Dear Mwangi,

RE: Approval of proposal by Faculty Biosafety, Animal use and Ethics committee

"Antimicrobial investigations, cytotoxicity evaluation and secondary metabolites of *Acokanthera schimperi* leaves crude extract".

Kamau Joe Mwangi J56/37447/2020

We refer to your MS.c proposal submitted to our committee for review and your application letter dated 21st December 2021.We have reviewed your application for ethical clearance for the study. The antimicrobial assay, toxicity evaluation of the plant using brine shrimp lethality test and phytochemical screening protocols meets the minimum standard of the Faculty of Veterinary medicine ethical regulation guidelines.

We also note that registered Veterinary surgeons will supervise the study.

We hereby give approval for you to proceed with the project as outlined in the submitted proposal.

Yours sincerely,

Kaluwa

Dr. Catherine Kaluwa, Ph.D
Chairperson, Biosafety, Animal Use and Ethics Committee,
Faculty of Veterinary Medicine,
University of Nairobi

Appendix II: NACOSTI License.

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **509560**

Date of Issue: **14/November/2022**

RESEARCH LICENSE

This is to Certify that Dr.. JOE MWANGI KAMAU of University of Nairobi, has been licensed to conduct research as per the provision of the Science, Technology and Innovation Act, 2013 (Rev.2014) in Narok on the topic: ANTIMICROBIAL INVESTIGATIONS, CYTOTOXICITY EVALUATION AND PHYTOCHEMICAL CONSTITUENTS OF Acokanthera schimperi (A.D.C) SCHWEINF LEAF CRUDE EXTRACTS for the period ending : 14/November/2023.

License No: **NACOSTI/P/22/21588**

509560

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

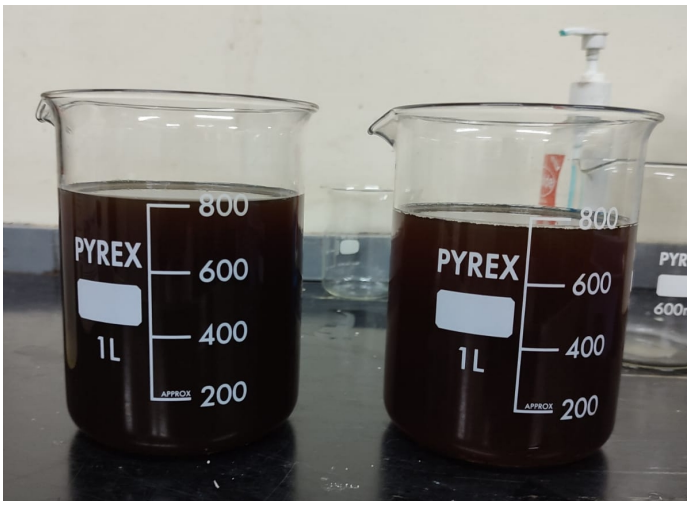
Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity of this document, Scan the QR Code using QR scanner application.

See overleaf for conditions

Appendix III: Photos taken in Narok forest during plant species collection.

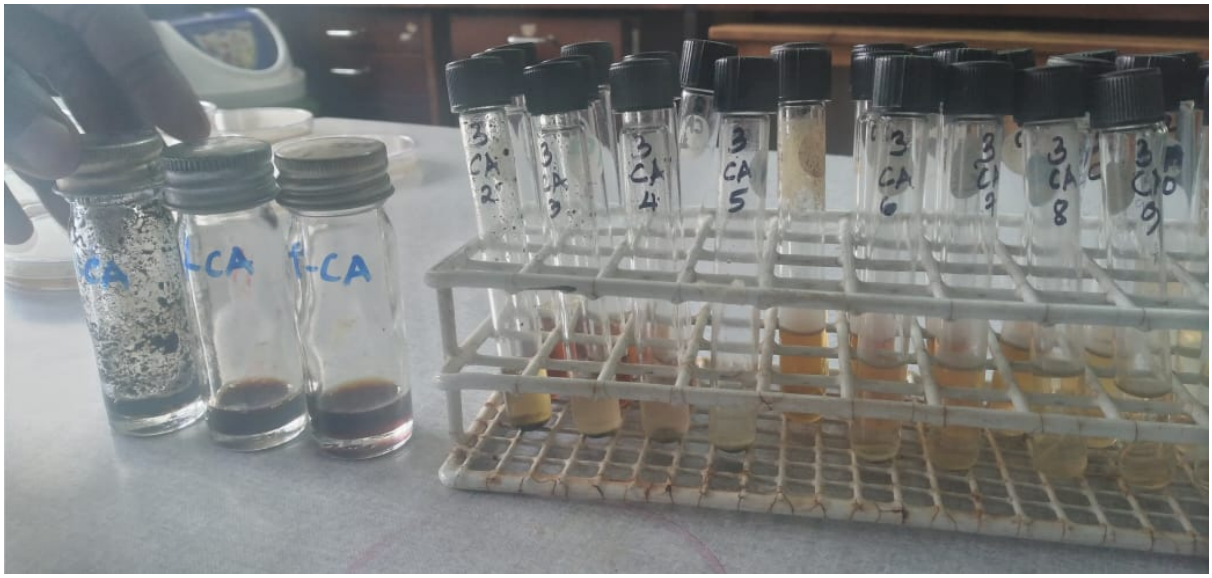
Appendix IV: Photos Taken during extraction.



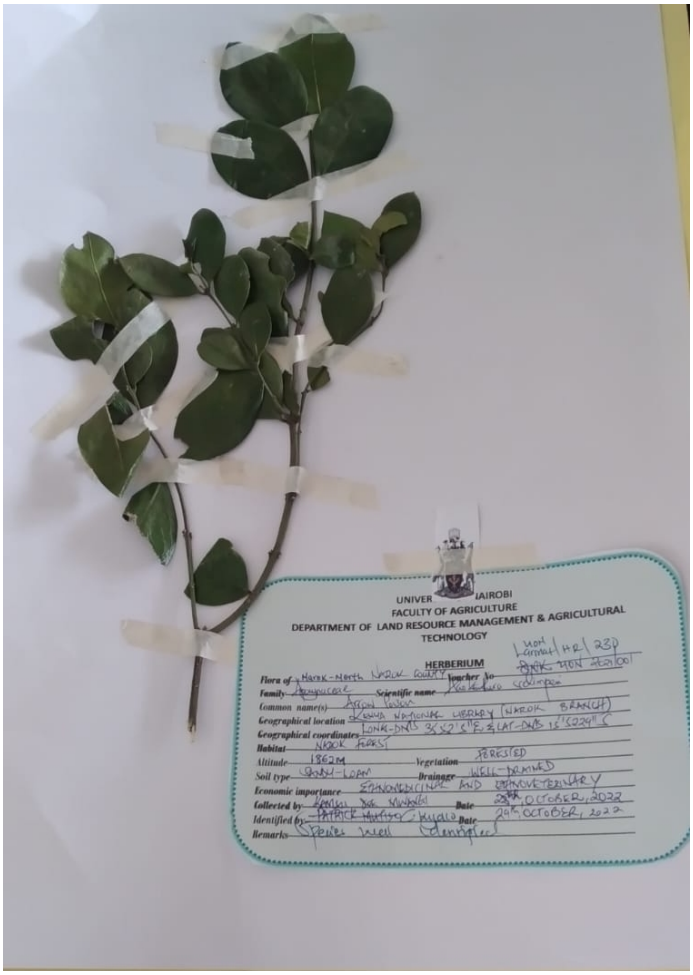
Photos determination of Minimum Inhibitory Concentration and

Appendix V;

Minimum Bacterial Concentration.


LCA
f-CA

Appendix VI; Photograph of Voucher specimen deposited at UON-Chiromo Herberium.



Appendix VII; Photos taken during cytotoxic evaluation during Brine shrimp Assay.

Appendix VIII; Photos taken during qualitative phytochemical determination