CHARACTERISATION, ANTIFUNGAL AND MOSQUITOCIDAL ACTIVITIES OF MORMODICA FOETIDA LEAF EXTRACTS AND THEIR FORMULATION INTO DROP PILLS.

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A THESIS SUBMITTED TO THE SCHOOL OF SCIENCE IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN CHEMISTRY, UNIVERSITY OF ELDORET, KENYA.

2015

DECLARATION

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DEDICATION

This thesis is dedicated to my daughter Perpetua, my mother-in-law, Mrs. Odongo and my parents Mr. and Mrs. Kitongo.

ABSTRACT

The upsurge in the prevalence of resistance to many synthetic antifungal agents and incidence of multidrug resistant mosquito-borne diseases has spurred scientists to research for new plant-based antifungal and mosquitocidal gents. Mosquitoes transmit serious human diseases that cause millions of deaths every year. This research investigated the mosquitocidal and antifungal activities of *Mormodica foetida*. Mosquitoes in the larval stage are attractive targets for insecticides. Botanicals contain various phytochemicals that are medicinally useful. In traditional medicine, people may discover their activity and prescribe them to their clients without prior knowledge of an effective dose. *M. foetida*, presents such potential of high medicinal and insecticidal value. Leaves from this plant are traditionally used to treat symptoms of malaria in parts of East Africa therefore it was screened for mosquitocidal and antifungal activities in this study. The plant leaves were collected in Kericho County, Kenya and extracted using decoction method. Liquid–liquid extraction of the resulting extract in Dimethyl Sulfoxide (DMSO) was done. IR, MS, and NMR were used to identify the compound in the extracts as an alkaloidal derivative of zeatin riboside. The antifungal activity of the water extract of *M. foetida* was investigated by disc diffusion assay method *in vitro* against *Candida albicans*, giving a zone of inhibition diameter (ZID) of 0.4mm. The DMSO extract gave higher % mortality values than the water extract on mosquito larvae. Supa kill was used as a reference in this study. The ground leaf powder was finally formulated into drop pills. Our data suggest that an alkaloidal derivative of zeatin riboside in DMSO extract of *M. foetida* has potential to be used in an ecofriendly approach for the control of mosquito infestations while its water extract is a potential fungicide.

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DEFINITION OF TERMS

Abortifacient: A substance that promotes abortion.

Aphrodisiac: A substance that enhances sexual wellness.

Decoction method: The process of boiling of medicaments in water for a given period of time to obtain a maximum amount of water-soluble components.

Drop pill: A solid formulation of the medicaments prepared by dropping method.

Ecbolic: A substance that induces contractions of the uterus, thus promote childbirth and / or abortion.

Excepients: A pharmacologically inactive substance used as a carrier for the active medicaments or to bulk up the formulation.

Pharmacology: A science that deals with origin nature chemistry effects and uses of drugs.

Phytochemistry: The study of secondary metabolic compounds found in plants in relation to their function.

Triterpene: A compound made up of six isoprene units

LIST OF ACRONYMS

1D NMR: One dimensional Nuclear Magnetic Resonance.

2D NMR: Two dimensional Nuclear Magnetic Resonance.

BuOH: Butanol.

COSY: Homonuclear Correlation Spectroscopy.

d: doublet.

DEPT: Distortionless enhancement by polarization transfer.

EtOAc: Ethyl acetate.

HMBC: Heteronuclear Multiple Bond Correlation.

HSQC: Heteronuclear Single Quantum Correlation.

IR: Infra red spectroscopy.

m: multiplet.

MS: Mass Spectroscopy.

NMR: Nuclear Magnetic Resonance.

s: singlet.

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CHAPTER ONE

INTRODUCTION

1.1 Background information

Natural products from plants are considered a rich source of bioactive chemicals with insecticidal properties (Das *et al*., 2001). Phytochemicals in plants have different activities such as ovipositor attractants, insect growth regulators, and repellants (Das *et al*., 2001). Over 80% of the world populations rely upon medicinal plants for their health care needs (Mwambete., 2009). The use of herbs as combination among herbal practitioners focuses on the synergistic action expected to take place by the traditional healer hence being able to give better results as compared to one herb and also treat more than one ailment (Odhiambo *et al.,* 2009). The Cucurbitaceae consist of about 120 genera and 850 species that are widely distributed (Jeffrey., 1990). Many species are commercially grown for their nutritive value and in some cases for medicinal purposes. In East and Central Africa, *M. foetida* is used to treat hypertension, diabetes mellitus, fever and especially symptoms of malaria (Hakizamungu *et al*., 1992). *Momordica foetida* is a perennial climbing vine native of tropical Africa. Its species name ("bad-smelling") refers to its unpleasant smell (Hyde and Wursten., 2011)**.** The leaves have a bitter taste but it is eaten. Mosquito species belonging to genera *Anopheles*, *Culex* and *Aedes* are vectors for the pathogens of diseases thus constitute a major public health problem. One of the approaches for control of these mosquito-borne diseases is the interruption of disease transmission by either, eradicating or preventing mosquitoes to bite human beings (Mohan and Ramaswamy., 2007).

1.2 Problem statement

Cucurbitacin (a flavones glycoside) has been isolated from *M. foetida* leaf extract and characterized by Mulholland *et al.,* (1996). Several other triterpenoids have also been isolated from non-polar and semi-polar extracts of *M. foetida* and other *Mormodica species* (Mulholland *et al*., 1996, Dictionary of Natural Products., 2014). The antimicrobial activity of some members in the genus *Momordica* have been studied and documented. However, there's a need to determine the antifungal activity of the *M. foetida* plant. In traditional medicine people often discover the activity of such botanicals and prescribe them to their clients without a prior knowledge of the effective dose. Since the active component in the plant material is not normally isolated the patients may end up taking large volumes of the concoction to achieve the desired dosage for the treatment of a particular disease. On the other hand vector control is facing a threat due to the emergence of resistance to synthetic insecticides. Although many plants have been reported to be useful as mosquitocidal agents, only a few botanicals have moved from the laboratory to the field use because they are poorly characterized. In most cases the active components are not isolated and most of the works are restricted to preliminary screening as described by Das *et al*., (2001).

1.3 Justification

This research is aimed at extracting alkaloids (nitrogenous bases) of *M. foetida* and to formulate them to drop pills which have the advantage of high bioavailability, high dissolution of in-dissolvable medicaments, increased shelf-life, and easy portability and hence would enhance effective patient compliance (Gao., 2005). Drop pill formulation increases the shelf-life of the medicament for up to two years. This is due to the

antioxidants contained in the excepients (Gao., 2005). In recent years, several diseases and microbial infections such as respiratory infections, bacterial meningitis, sexually transmitted as well as hospital acquired infections have shown considerable resistance to a number of antimicrobial agents, such as penicillin, ampicillin, and flouroquinolones. There is an increasing trend in the emergence of resistance to antimicrobial agents, not only due to the poor quality drugs, patient non-compliance, and irrational use of antimicrobial agents, but also to spontaneous mutations within the microbial populations (Mwambete., 2009). Antifungal assay in this study was done to verify the activity of the medicament in the leaf of *M. foetida*. Mosquitoes are the most important single group of insects in terms of public health which transmit a number of diseases for instance malaria, filariasis, dengue (Das *et al.,* 2001). Repeated use of synthetic insecticides for mosquito control has disrupted natural biological control systems and led to the resurgence in mosquito populations (Das *et al*., 2001). The use of insecticides has also resulted in the development of resistance, undesirable effects on non-target organisms and fostered environmental and human health concern which necessitates a search for alternative control measures (Das *et al*., 2001).The leaf extract of *M. foetida* herein was screened for mosquitocidal activity. Mosquitoes in the larval stage are attractive targets for pesticides because they breed in water and they are easy to deal with in this habitat. MS, NMR and IR were used to identify the alkaloid derivative of zeatin riboside in the leaf extracts.

1.4 Objectives

1.4.1 General objective

To determine the compounds present in the leaf extract of *M. foetida* and study their antifungal and mosquitocidal activities and formulate the compound into drop pills.

1.4.2 Specific objectives

i. To identify and determine the nature of compound in *M. foetida* leaf extracts using IR, NMR, and MS.
ii. To formulate *M. foetida* leaf extracts into drop pills, regulate their weight variation, color and determine their solubility in water at 37 ^{0}C and at 100 ^{0}C.
iii. To determine the mosquito larvicidal activity of the acid-base leaf extract.
iv. To determine the antifungal activity of acid –base extracts of *M. foetida* against *C. albicans*.

1.5 Hypotheses

The study was conducted guided by the following hypotheses:

i. IR, NMR, and MS can effectively determine the chemical structure of the compound in the extract of *M. foetida.*
ii. The dropping method can formulate *M. foetida* drop pills of the required quality.
iii. The acid-base leaf extract of *M. foetida* has significant mosquito larvicidal activity.
iv. *M. foetida* has significant antifungal activity against *C. albicans*.

CHAPTER TWO

LITERATURE REVIEW

Momordica foetida, a plant in the family *Cucurbitaceae* is of nutritional and medicinal value. The leaves of this plant although having a bitter taste and foetid smell when crushed are collected from the wild and eaten after boiling as a vegetable (Ruffo., 2002). In Malawi the fruit is also used as bait to trap birds, In Gabon the leaves are soaked, dried in the sun, and used to stuff cushions. In Tanganyika the fruit pulp is believed to be poisonous to weevils, moths, ants and is used as an insect repellant.

2.1 Botanical description of *M. foetida.*

The plant is a dioecious, perennial herb, trailing or climbing with simple or bifid tendrils; stem is long, with dark green flecks when young, woody when old, rooting at the nodes (Plate 2.1).

Plate 2.1 A photograph showing the *M. foetida* plant on a fence (Source: Author, 2010).

Leaves are alternate, simple; stipules absent; petiole 1.5–17 cm long; blade broadly ovate-cordate to triangular-cordate (**Plate 2.2**). Flowers are unisexual, regular, calyx with obconic tube petals free, obovate-lingulate, white, pale yellow to orange-yellow, 3 with scales inside at base; male flowers are together in fascicles on peduncle with 3 stamens, anthers coherent in centre of flower; female flowers solitary in leaf axils, with inferior, ovoid ovary, stigma 3-lobed **(Plate 2.2).**

Plate 2.2 A photograph of the *M. foetida* leaves and the opened flower (Source: Author, 2010).

The fruit is a long-stalked, ellipsoid berry orange when ripe, densely and softly spiny, dehiscing with 3 valves and exposing the many seeds embedded in scarlet pulp **(Plates 2.3 and 2.4)**. Seeds oblong, flattened, brown, testa sculptured, margins 2-grooved (**Plate 2.5)** respectively.

Plates 2.3 and 2.4 Photographs showing the ripe and the open fruit respectively (Germer., 2010).

Plate 2.5 A photograph of the opened fruit and seeds (Germer., 2010).

2.2 Habitat of *M. foetida.*

The plant grows in forest edges and similar habitats (including undisturbed and cultivated land), woodland, and wooded grassland (Bosch *et al.*, 2004). *M. foetida* occurs in forest edges and clearings, margins of swamps and on disturbed ground as a weed and colonizer, up to 2400 m altitude and it is widespread in tropical Africa and in South Africa (Jeffrey., 1990).

2.3 Phytochemistry.

A survey for compounds isolated previously from the Momordica genus using the CHEMNETBASE, Dictionary of Natural Products online database (http://www.chemnetbase.com) showed that 229 compounds have been reported. Amongst the classes of compounds isolated include cucurbitacin tritepernoids represented by cucurbita-5, 24-diene-3, 7, 19, 23-tetrol (Figure 2.1) isolated from M. *charantia* and their glycoside derivatives represented by Karaviloside IV (Figure 2.2) also isolated from M. *charantia*. Aliphatic glycosidic amide (nitrogen containing compound) has also been isolated from M. *charantia* (Figure 2.3) (Dictionary of Natural Products., 2014). Oleanolic and ursane type triterpenoids have also been reported from members of Momordica *genus* (Figure 2. 4 and 2.5 respectively). Also isolated from this genus are pimarane type diterpenoids represented by 15-pimarene-9, 17, 18-triol (Figure 2.6), stigmastane represented by structures shown in figure 2.7. A flavone (Figure 2.8), a pyrimidine derivative (Figure 2.9), zeatin (Figure 2.10) and its riboside derivative (Figure 2.11) have also been reported from Momordica (Dictionary of Natural Products., 2014). Bitterness in most plants is due to the presence of alkaloids, saponins and cardiac glycosides. The bitterness in the leaves could be attributed to the presence of cucurbitacins which are a group of bitter-tasting, highly oxygenated, mainly tetracyclic plant substances derived from the cucurbitane skeleton (Miro., 1995).

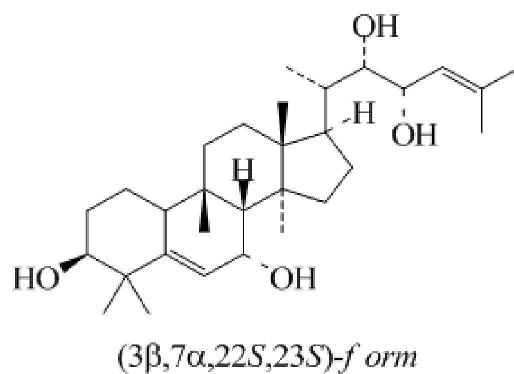


Figure 2.1 Structure of cucurbita-5, 24-diene-3, 7, 19, 23-tetrol a representative of a cucurbitacin triterpenoid. (Dictionary of Natural Products., 2014).

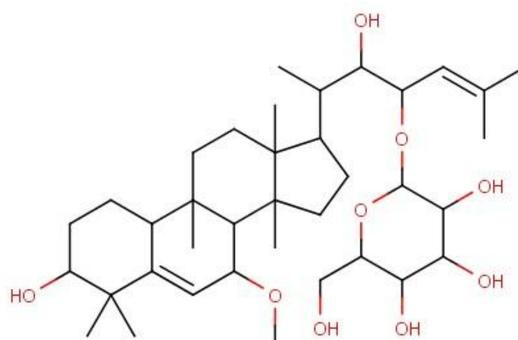


Figure 2.2 A structure of Karaviloside IV, a representative of cucurbitacin glycoside isolated from *Momordica* genus (stereochemistry not shown but is similar to that shown in Figure 2.1 and the glycoside is β□D-glucopyranose) (Dictionary of Natural Products., 2014).

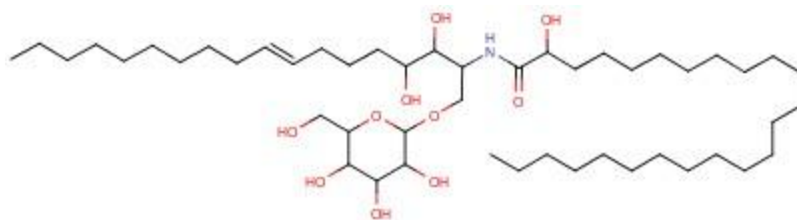


Figure 2.3 A glycosidic aliphatic amide isolated from a member of *Momordica genus.* (Dictionary of Natural Products., 2014).

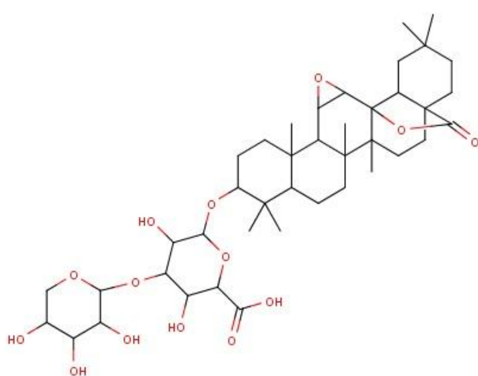


Figure 2.4 A structure showing a glycosidic oleanolic acid ester derivative from *Momordica* (Dictionary of Natural Products., 2014).

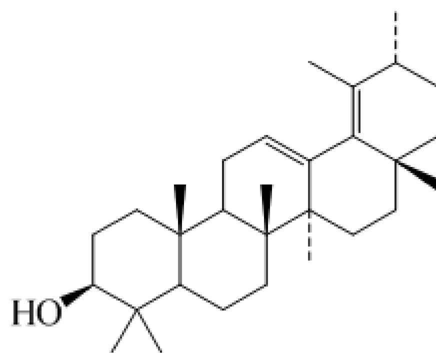


Figure 2.5 Ursane type triterpenoid from *Momordica genus* (Dictionary of Natural Products., 2014).

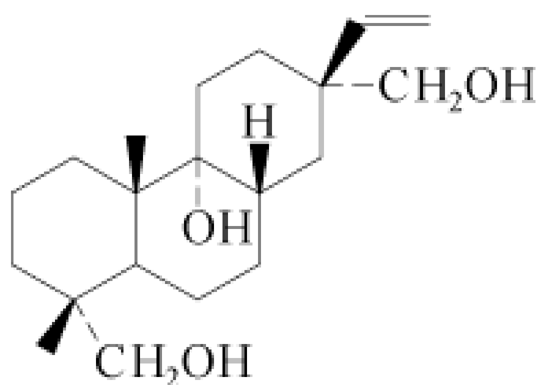


Figure 2.6 Pimarane type diterpenoid from *Momordica* genus (Dictionary of Natural Products., 2014).

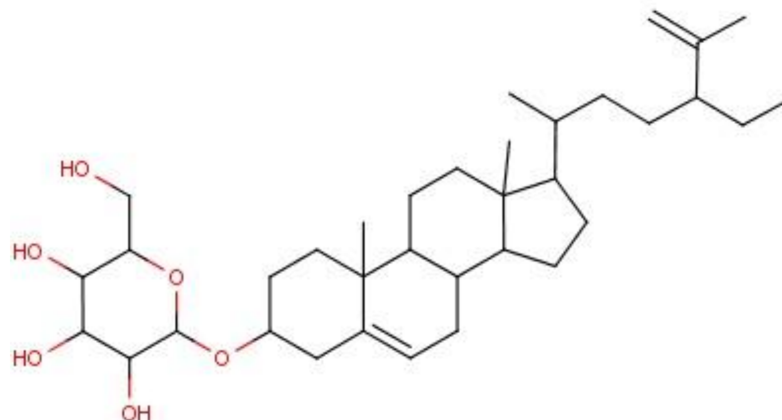


Figure 2.7 Stigmastane derivative from *Momordica* genus (Dictionary of Natural Products., 2014).

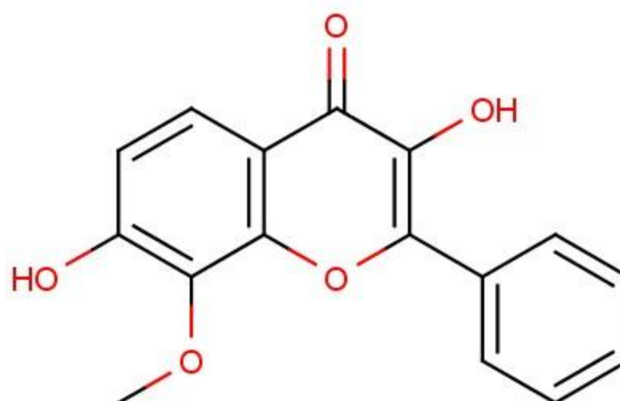


Figure 2.8 An example of flavone, 3, 7-Dihydroxy-8-methoxyflavone, isolated from *M. dioca* (Dictionary of Natural Products., 2014).

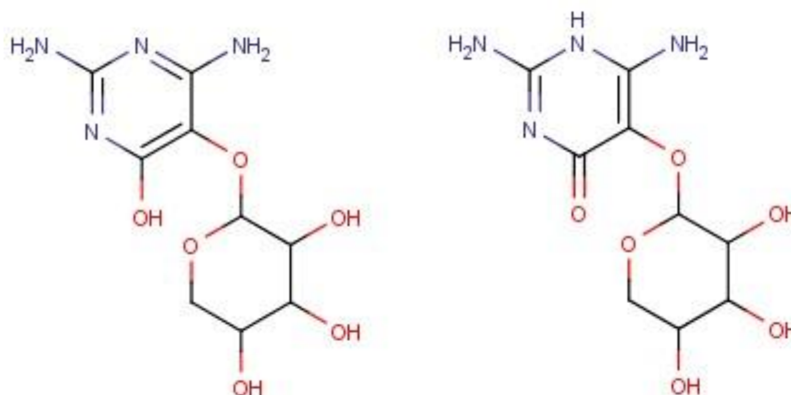


Figure 2.9 2, 4-Diamino-5,6-dihydroxypyrimidine-5-*O*-arabinopyranoside (Dictionary of Natural Products., 2014).

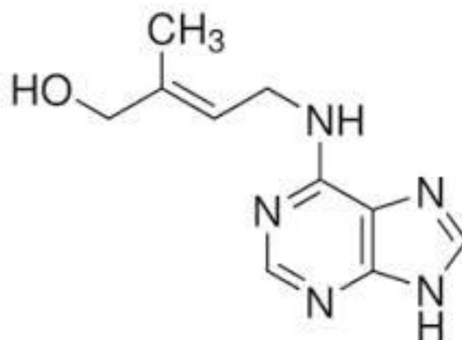


Figure 2.10 Structure of zeatin, a nitrogen containing hormone, isolated from *Momordica* genus (Dictionary of Natural Products., 2014).

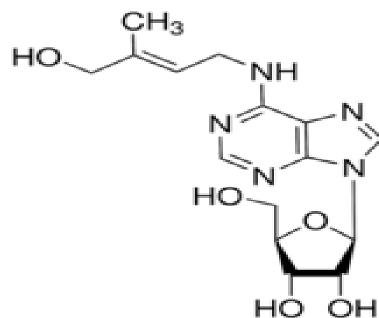


Figure 2.11 Structure of zeatin riboside, nitrogen containing glycosylated hormone, isolated from *Momordica* genus (Dictionary of Natural Products., 2014).

2.4 Uses of parts of *M. foetida.*

Various parts of *M. foetida* have been studied for their medicinal uses. In this study they are categorized as either traditional or modern.

2.4.1 Traditional medicinal uses.

Traditional medicinal uses are numerous and many are shared with other representatives of *Momordica* genus. Some of the members of this genus of medicinal value are *M. balsamina* (Balsam apple) whose fresh ripe fruit is used to cure abdominal pains, painful and profuse menses with labor-like pains. *M. charantia* (*Bitter* gourd) has been found to cure cholera, cramps, thirst and prostration *M. involucrate* has been indicated for stomach complaints. *M. foetida* is used to relieve constipation and cure worm infections (Ajith, 2012). The juice of crushed leaves of *M. foetida* is used to relieve cough, stomach-ache, intestinal disorders, headache, earache and toothache. Skin problems caused by smallpox, boils, and malaria were treated with crushed leaves. The plant was further used as an ecbolic, aphrodisiac and abortifacient (Jeffrey., 1967). A study carried out in the Kenyan

Lake Victoria basin by Odhiambo *et al.*, 2009 revealed high usage of herbal drugs in combined proportions to treat ailments such as malaria, sexually transmitted infections, and typhoid. *T. asiatica* (Rutaceae), R. *taddo* (Rhamnaceae), *P. falcatus* Podocarpaceae), *M. foetida* (Cucurbitaceae) and *Aloe sp*. (Aloacaeae) are some of the plants used by local communities in the Kenyan Lake Victoria Basin as a combined proportion to treat malaria.

2.4.2 Modern medicinal uses.

2.4.2.1 Antimicrobial activity.

Odeleye *et al*., (2009) screened *M. foetida* leaves for antimicrobial activity against 32 bacterial strains for both standard and isolates. Thus, ethyl acetate and chloroform fractions were chosen for further studies due to their higher antimicrobial activity with minimum inhibitory concentration (MIC) values for 32 bacterial strains ranging from 0.156 to 2.5 mg mL^{-1}. A detailed phytochemical investigation resulted into isolation of four curcubitane triterpenoids and flavonoids from chloroform and ethyl acetate fractions respectively. The chemical structures of the isolated compounds were established using UV, IR, MS, ^{1}H, ^{13}C, and 2D NMR spectroscopic data. Antimicrobial investigations were carried out on the isolated compounds against 25 bacterial strains of which 3β,7β-dihydroxyl-cucurbita-5,23,25-trien-19-al followed by Kaempferol-*3*-*O*-β-D-glucopyranoside displayed minimum inhibitory concentration (MIC) values for 25 bacterial strains ranging from 7.8 to 250 μg mL^{-1}.

Waako *et al.,* (2005) compared two plants *C. helicacabum* and *M. foetida* screened for *in vitro* and *in vivo* antimalarial activity. Using the nitro tetrazolium blue-based parasite

lactate dehydrogenase assay as used by Makler *et al.,* (1993), water extracts from the two plants were found to have weak *in vitro* antiplasmodial activity with 50% inhibitory concentrations (IC_{50}s) greater than 28.00 µg/ m L. *In vivo* studies of water extracts from the two plants showed that *M. foetida* given orally in the dose range of 10, 100, 200 and 500 mg/kg twice daily prolonged survival of *Plasmodium berghei* infected mice from 7.0+/-1.8 to 17.9+/-1.8 days. The water extract of *Cardiospermum helicacabum* was toxic to mice, none surviving beyond day 4 of oral administration, with no evidence of protection against *Plasmodium berghei* malaria. The study emphasized the discrepancy that might be found between *in vitro* and *in vivo* testing of plant-derived antimalarial extracts and the need to consider *in vitro* antiplasmodial data with this in mind.

Sonja *et al*., (2007) also studied the *in vitro* antimalarial activity of the leaves from *M. foetida* that are traditionally used to treat symptoms of malaria in parts of East Africa. Using an [^{3}H] hypoxanthine-incorporation assay the antiplasmodial activity of hydrophilic and lipophilic extracts against the chloroquine-sensitive strains poW and the multiresistant clone Dd2 of *Plasmodium falciparum* was determined. The petrol ether/ethyl acetate extract showed significant activity with IC_{50} values of 7.3 µg/mL (poW) and 13.0 µg / mL (Dd2). Phytochemical analysis led to the isolation of a number of phenolic glycosides, such as eriodictyol-5, 7, 4'-trihydroxyflavanone-, kaempferol- and 5, 7-dihydroxychromone-7-O-D-glucopyranoside, not previously known from *M. foetida*.

Mwambete., (2009) evaluated the antimicrobial activity of *M. charantia* extracts on reference strains and microorganisms isolated from clinical specimens. Petroleum ether and methanolic crude extracts of fruits and leaves of the plant were studied for

antimicrobial activity using the disk diffusion method on four reference microorganisms (*P. aeruginosa, E. coli, C.albicans* and *S. aureus*; and four clinical strains of *K. pneumoniae, P. vulgaris, S. typhii* and *C. neoformans.* Antimicrobial activity was observed against all the tested microorganisms with exception to *P. mirabilis* and *C. neoformans.* Methanolic crude extracts exhibited relatively broader antimicrobial spectrum of activity than petroleum ether extracts with as low a concentration as 0.075 mg /µL. Methanolic fruit crude extract displayed the broadest antimicrobial spectrum by inhibiting majority (75%) of the tested microorganisms.

Odhiambo *et al.,* (2009) investigated the anti-aspergillus and anti-candida efficacy of crude extracts of five plants used in combination to treat malaria. *T. asiatica* (root)*, R. staddo* (root), *M. foetida* (shoot), *P. falcatus* (bark) and an *Aloe sp.* (succulent leaves) used by traditional health practitioners in the Kalenjin community. The plants were extracted using water and dichloromethane/methanol (1:1) and the crude extracts tested for *in vitro* antifungal activity singly and in combinations against *A. niger* and *C.albicans.* Dichloromethane/methanol extracts of *P. falcatus* showed the highest activity (77.77% inhibition) against *A. niger* while *M. foetida* showed the highest activity (77.78% inhibition) against *C. albicans. Aloe sp.* showed no activity against *A. niger* when tested singly (Odhiambo *et al.,* 2009). Although the main aim of the study was to conduct an ethno medicinal survey on plants used to treat fungal infections in the area and thereafter establish their claimed therapeutic activity, it was predicted that diseases such as malaria had similar signs and symptoms to those of systemic fungal infections. Odhiambo and co-authors (2009) in their study, report the antifungal activity of crude extracts used in combined proportions against *C.albicans* and *A. niger*.

According to Subhashchandra *et al.,* (2010) *M. charantia* contains charantin, steroidal saponin, momordicosides, carbohydrate, mineral matters, ascorbic acid, alkaloids and glucosides. Subhashchandra and co-workers (2010) carried out an isolation, purification and characterization of charantin from fruit of *M. charantia* Linn. The isolated charantin was further characterized with the help of ultraviolet spectroscopy, thin layer chromatography, fourier transform infra red spectroscopy, mass spectroscopy and proton-nuclear magnetic resonance spectroscopy. The antibacterial activity of charantin was tested using Agar Diffusion (Cup Plate) method. The minimum inhibitory concentration (MIC) of crude extracts for various organisms was 0.2 mg /mL.

2.4.2.2 Mosquitocidal activity.

Das *et al*., (2001) In view of the recent interest in developing plant-based insecticides as an alternative to chemical insecticides, undertook a study to assess the larvicidal potential of the fruit wall extracts of *M. charantia* against two species of mosquito vectors, *A. stephensi* and *C. quinquefasciatus*. Among the extracts tested, petroleum ether (LC_{50} = 27.60; 17.22 ppm and 41.36; 15.62 ppm) extract was found to be more effective than carbon tetrachloride (LC_{50} = 49.58; 16.15 ppm and 80.61; 27.64 ppm) and methanol (LC_{50} = 142.82; 95.98 ppm and 1,057.49; 579.93 ppm) extracts towards anopheline and culicine larvae after 24 and 48 h of exposure respectively. Thus, all fruit wall extracts of *M. charantia* are toxic to both larval species.

Rahuman and Venkatesan., (2008) studied the larvicidal activity of crude hexane, ethyl acetate, petroleum ether, acetone, and methanol extracts of the leaf of five species of cucurbitaceous plants. *C. colocynth's, C. indica, C. sativus, M. charantia*, and *T. anguina*, was tested against the early fourth in star larvae of *A. aegypti* and *C.*

quinquefasciatus (Diptera: Culicidae). The larval mortality was observed after 24 h of exposure. All extracts showed moderate larvicidal effects; however, the highest larval mortality was found in petroleum ether extract of *C. colocynthis*, methanol extracts *of C. indica, C. sativus, M. charantia*, and acetone extract of *T. anguina* against the larvae of *A. aegypti* (LC_{50}â=â74.57, 309.46, 492.73, 199.14, and 554.20Â ppm) and against *C. quinquefasciatius.*

Savitha *et al*., (2006) tested the methanolic extracts of leaves and seeds from the chinaberry tree, *Melia azedarach* L. (Meliaceae) against mature and immature mosquito vector Anopheles stephensi Liston (Diptera) under laboratory condition. The extract showed strong larvicidal, pupicidal, adulticidal, antiovipositional activities, repellency and biting deterrency. The *M. azedarach* seed and leaf extracts were used to determine their effect on *A. stephensi* adults and their corresponding oviposition and consequent adult emergence in comparison with the control. The seed extracts showed high bioactivity at all doses, while the leaf extracts proved to be active, only in the higher dose.

Mohan and Ramaswamy., (2007) attempted to analyze the larvicidal effect of the leaf extract of a vastly grown (in the hilly regions of the Nilgiris district) weed plant, *A. adenophora* on two important mosquito species, *A. aegypti* and *C. quinquefasciatus*. The larval mortality of fourth in star larvae of *A. aegypti* and *C. quinquefasciatus* after 24 h of treatment were observed separately in control, 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 ppm concentrations of the leaf extract(acetone) of *A. adenophora*. Based on the probit analysis, the 24 h LC_{50} value of the leaf extract of *A.adenophora* was found to be 356.70 ppm for *A. aegypti* and 227.20 ppm for *C. quinquefasciatus.* When compared to

neem, the leaf extract of *A. adenophora* was more toxic to both *A. aegypti* and *C. quinquefasciatus* and could be effectively used for the control of mosquito larvae.

2.4.2.3 Antihelminthic.

A study by Wasswa and Deogracious., (2007) identified twenty-one plants through preliminary field surveys and seven were selected for *in vitro* anti-helminthic activity against *Ascaris suum*. Of the seven plants that were initially screened, five gave appreciable positive results while two did not. Their research findings showed that *T. riparia, C. occidentalis, C. papaya, M. foetida* and *E. abysinnica* may be of value in the treatment of helminthiasis; whereas *M. oleifera* and *C. sativa* are probably ineffective or of limited value for the same purpose.

2.4.2.4 Laxative effect.

According to Odusote and Awaraka., (2004) the dried extract of *M. foetida* was granulated with cornstarch mucilage to produce free flowing granules. Capsules containing 500 mg of the granules were hand filled then evaluated in terms of uniformity of weight and disintegration. Phytochemical analysis of the plant extract showed the presence of alkaloids, saponins and cardenolides. Bioassay of the extract was conducted to verify the laxative effect. Their result indicated that the cumulative number of faeces per mouse per hour for different doses increased as the concentration of the medicament increased. In conclusion, *M. foetida* leaf extract was found to be a potential laxative and could be formulated into a solid –dosage form to enhance patient compliance.

2.4.2.5 Antioxidant activity.

Acquaviva *et al*., (2013) studied the antioxidant activity of *M. foetida*. Diabetes mellitus is a group of metabolic disorders characterized by alterations in carbohydrate, fat and protein metabolisms associated with absolute or relative deficiency of insulin secretion and /or insulin action. Studies indicate that hyperglycemia triggers the generation of free radicals and oxidative stress in capillary endothelial cells in the retina, mesangial cells in the renal glomerulus and neuron cells in the peripheral nerves (Brownlee., 2005). Treatment strategies that focus on decreasing oxidative stress as well as enhancing antioxidant defense systems might present important options for the treatment of diabetic complications. Hence compounds with both antihyperglycemic and antioxidative properties would be useful antidiabetic agents (Baynes., 1995).

2.5 Alkaloids.

Alkaloids are a heterogeneous group of compounds and consequently it's difficult to define them accurately. The word alkaloid is derived from the term "vegetable alkali" used originally to describe bases of botanical origin. Most alkaloids are basic, nitrogen–containing heterocyclic compounds derived from higher plants often having marked physiological activity (Gao., 2005).

The basic nitrogen of alkaloids may be primary, secondary, tertiary or quaternary. The alkaloids are usually classified mainly on structural grounds because of heterocyclic ring systems common to each group (e.g. pyridine or quinolone systems), but sometimes also because of the occurrence in certain plant families e.g. alkaloids containing an indole nucleus are widely distributed in the plant world while alkaloids from the Amaryllidaceae form a distinct group (Gao., 2005)

2.6 Extraction of alkaloids.

Extraction is the process of obtaining active components with appropriate solvents. The purpose of extraction, refinement and separation are to: extract active principle or active fraction, reduce dosage of use and increase curative effects. A suitable extraction solvent extracts the active constituents to the maximum and the inactive constituents to the minimum, does not react with active constituent, does not affect the stability and curative effect of the active constituent, and is safe, non-toxic and cheap. Most methods exploit the property of most alkaloids to be soluble in organic solvents but not in water, and the opposite tendency of their salts. Most plants contain several alkaloids. Their mixture is extracted first and then individual alkaloids are separated by column chromatography. They are separated from their mixture using their solubility differences in certain solvents and different reactivity with certain reagents or by distillation. Plants are thoroughly ground before extraction. Most alkaloids are present in plants in the form of salts of organic acids (Gao., 2005). The extracted alkaloids may remain salts or change into bases (Gao., 2005). In the acidic extraction, the raw plant material is processed by a weak acidic solution to a protonated alkaloid which when basified is converted to alkaloids.

2.6.1.1 Extraction with acid.

The extraction is achieved with 0.1-1% sulphuric acid, hydrochloric acid or acetic acid. The main objective of adding acid is to facilitate extraction of alkaloids, increase stability of some kinds of alkaloids, facilitate extraction of organic acid by making it free acid, eliminate impurity that can‘t dissolve in acid (Gao., 2005). The amount of acid should be definite to maintain a certain pH value. Excess acid may lead to unwanted hydrolysis of some kind of constituents such as glycosides. Aqueous solutions of alkali can dissolve

internal ester, anthraquinones and their glycosides, organic acids, resin and certain proteins to increase impurities. Ammonia water is the most commonly used alkali which brings about a little decomposition of constituents (Gao., 2005).

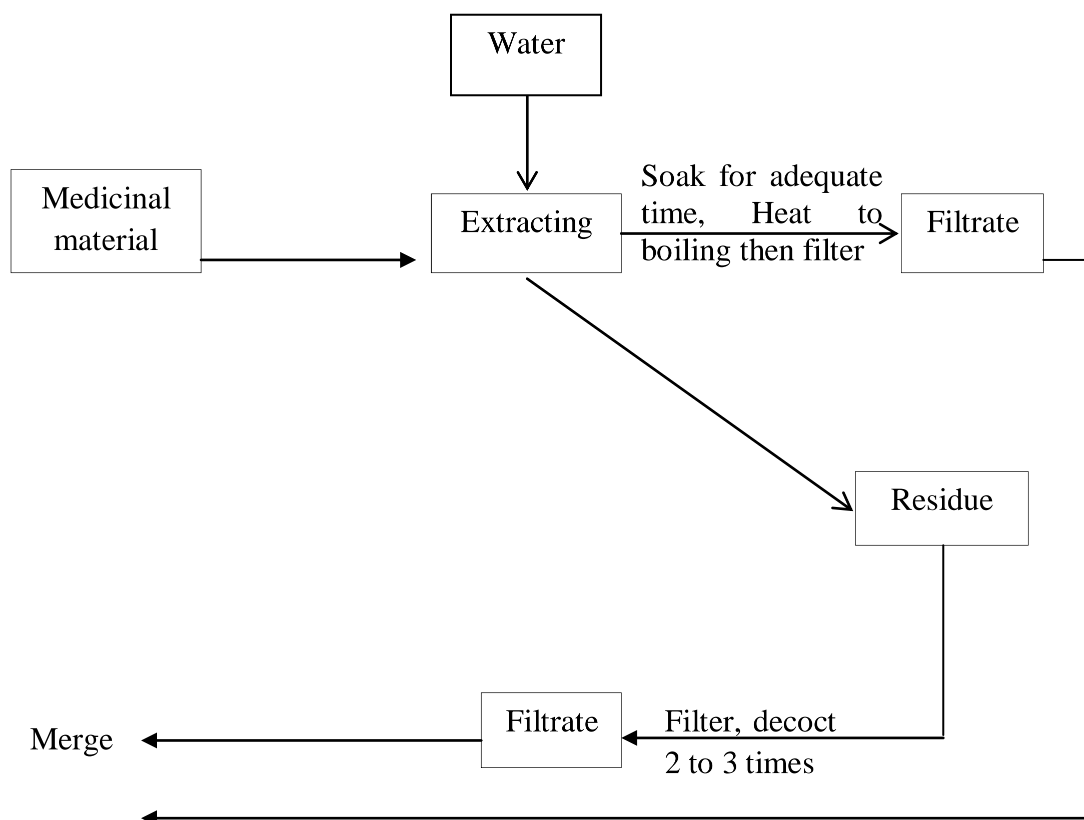
2.6.1.2 Liquid-liquid extraction.

Simple liquid-liquid extraction is still valued in chemical research. In this method, the mixture is separated by the difference in distribution of coefficients in two phase solvent using a separating funnel (Gao., 2005). Liquid-liquid means that two immiscible liquids are used in the extraction procedure. Extraction is such that some compounds are more soluble in the organic layer and other compounds are more soluble in the aqueous layer.

DMSO is a polar aprotic solvent which is frequently used in chemical reactions involving salts (Gao., 2005). The advantages DMSO solvent include its ability to tolerate relatively strong bases due to its weakly acidic nature, slow evaporation at normal atmospheric pressure because it has a high boiling point and effective sample management and high throughput screening operations in drug design due to its ability to dissolve many kinds of compounds (Gao., 2005).

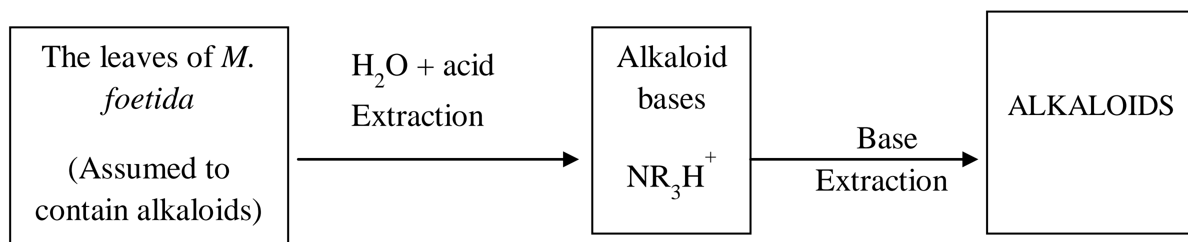
2.6.1.3 Decoction method.

The process involves boiling medicinal materials with water for a certain period of time to obtain a maximum amount of water soluble components. The method is suitable for medicinal materials which can keep stability in wet, hot circumstance, not volatile in steam (Gao., 2005). Using this method, illustrated in Scheme 2.1, more constituents can be extracted from the medicinal material.



Scheme 2.1 An illustration of the decoction method.

A decoction extraction (hot water-acid extraction) technique followed by base hydrolysis was used to isolate compounds present in the leaves of *M. foetida*. Based on the extractability of the hot water and acidic media mainly glycosylated nitrogenous compounds were isolated (Scheme 2.2).



Scheme 2.2 A scheme showing the extraction of alkaloids from *M. foetida*.

Glycosylated alkaloids were isolated because hot water (a polar solvent) will solubilize polyhydroxylated compounds whereas hydrochloric acid would form nitrogenous bases due to presence of nitrogen atoms. Finally, treatment of the alkaloid bases with NH_4OH resulted in alkaloids.

2.7 Spectroscopic analyses.

The spectroscopic techniques employed in the study are IR, MS, ^{1}H NMR and 2D NMR techniques.

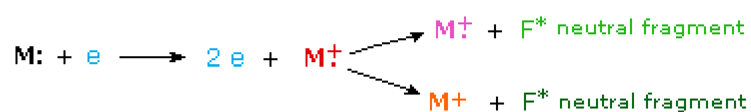
2.7.1 Infrared spectroscopy.

Electromagnetic radiation in the infrared (IR) region of the spectrum has the correct energy to cause bonds in a molecule to stretch and bend (Kemp, 1991). Individual functional groups have a characteristic absorption in the IR region (Kemp, 1991). The absence of an absorption in the IR spectrum of a compound can be important. For example, if an oxygen-containing compound shows no absorption in the C=O region (1680-1750 cm^{-1}) or in the O-H region (2500 - 3650 cm^{-1}) of the IR spectrum, the compound is likely to be an ether (Kemp, 1991). The region of an infrared spectrum below about 1500 cm^{-1} is termed the *fingerprint region*. Absorptions in this region

usually result from vibrations of the molecule as a whole and no two compounds have exactly the same absorption in the fingerprint region (Kemp, 1991).

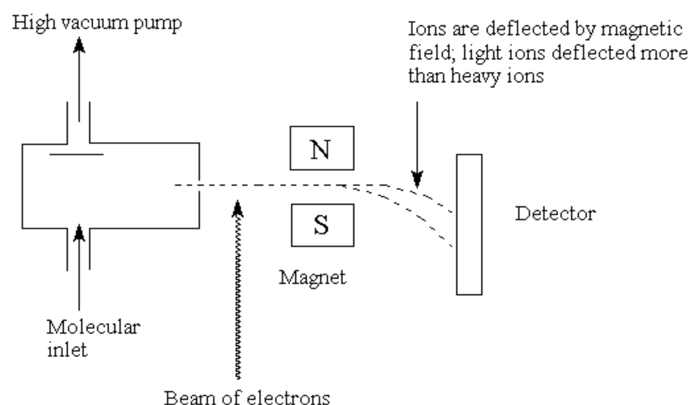
2.7.2 Mass spectrometry.

Mass spectrometry is used to determine the molecular mass of an organic compound (Lide., 2002). When a small sample of the compound is vaporized under very low pressure and high temperature and the vapor irradiated with a beam of high energy electrons (4000 - 6000 kJ mol^{-1}), it ionizes it by knocking away one of the molecular electrons (either bonding or non-bonding) (Lide., 2002) . This leaves behind a molecular ion (colored red in Scheme 2.3). Residual energy from the collision may cause the molecular ion to fragment into neutral pieces (colored green) and smaller fragment ions (colored pink and orange). The molecular ion is a radical cation, but the fragment ions may either be radical cations (pink) or carbocations (orange), depending on the nature of the neutral fragment(Kemp, 1991).



Scheme 2.3 A scheme showing the formation of a molecular ion.

The cations are accelerated through an electric field into a magnetic field where they are deflected as shown in Scheme 2.4. The ions are characterized by their mass (*m)* to charge (*z*) ratio (*m/z*) (Lide., 2002).



Scheme 2.4 A scheme showing a summary of the processes in a mass spectrophotometer.

The mass spectrum of a compound typically shows a number of signals and the peak at highest *m/z* (*molecular ion*) usually corresponds to the mass of the whole molecule. The signals with lower *m/z* are fragment ions and can provide some structural information (Lide., 2002).

2.8 Drop pills.

Drop pills are formulations prepared by blending the extract of medicinal materials and bases homogenously under thermal conditions, dripping the mixture into immiscible condensate, condensing them and allowing them to contract fully (Gao.,2005). The drop pills were formulated on a dropper using the dropping method. The ratio of excepients to medicaments was based on trial and error to determine the best ratio.

Drop pills consist of drugs and excepients. The excepients should be such that they do not interact with the principal drug, are physically and chemically stable, not harmful to

human beings and be of low cost. The excepients should be biocompatible and biodegradable in the active component. The excepients for drop pills are synthetic polymers –polyvinylpyrrolidine (PVP), polyethylene glycol (PEG) (Gao., 2005).

The moulding technique of drop pills formulation in such that a highly finished product and of good quality is achieved. The moulding probability of drop pill is usually used as the evaluation to choose the best dropping condition (Zhong., 2010).

2.8.1 Advantages of excepients.

i. Are pharmacologically inactive and therefore used as carriers of active medicinal ingredients.
ii. They are carriers of active ingredients especially when they are not easily administered or absorbed in the human body.
iii. They are used to bulk up formulations that contain very potent active ingredients to allow for convenient and accurate dosage.
iv. Used to stabilize active ingredient that denatures, falls out of solution or sticks to the side of the container on purification.

2.9 Disc diffusion method of drug sensitivity.

Circular paper discs 8 mm diameter are cut out from What man No. 1 filter paper using a paper punch and each dipped in a known concentration of the plant extracts for about 2 min, then gently transferred to the centre of the inoculated agar media. Petri dishes inoculated with microbial are kept for incubation for 24 h at a given temperature. The diameters of growth inhibition zones are measured using a ruler and compared to the control disc that has been dipped in dissolving solvent to nullify the effect of the solvent on the growth of the test organisms.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Chemical Reagents.

Hydrochloric acid (12M), Ammonia (0.025M), Dimethyl sulfoxide Polyethylene glycol - 6000, was obtained from Unilab, Kobian. Water was distilled at the Chemistry Laboratory in University of Eldoret. All the reagents used were general purpose. Potato dextrose Agar was obtained from H. Media Laboratories Ltd, India, Nystatin and liquid paraffin were obtained from Dawa Ltd, Nairobi and Supakill.

3.2 Equipment.

All the weights were taken on sensitive Sartorious analytical balance, Bosch PE 625 Model. Infrared spectra were recorded on a Shimadzu Model 408 Spectrophotometer; Rotary evaporator was the Buchi EL 130 Model available at the analytical Chemistry Laboratory in the University of Eldoret. Mass Spectrometry (MS) was carried out at the University of Surrey (Guildford, United Kingdom). 1D and 2D NMR spectra were recorded using a 500 MHz Bruker AVANCE NMR instrument at University of Surrey (Guildford, United Kingdom). The drop pills were formulated on a dropper at the University of Eldoret.

3.3 Other materials.

The plant sample, *M. foetida* leaves and Mosquito larvae.

3.4 Method.

The methods used in the study were acid –base extraction of alkaloids, dropping method of drop pill formulation, antifungal and mosquito larvicidal assays.

3.4.1 Sample collection and preparation.

Plant leaves were collected in Sitotwet in Kericho County, Kenya in November 2010. The plant was identified, authenticated and a voucher specimen, ANN/KAK/06/10/001, in duplicate was deposited in the herbarium at the University of Eldoret. Leaves of *M. foetida* were air dried at room temperature for 3 weeks. The leaves were subsequently ground into fine powder using a mortar and pestle yielding 300 g and stored in a plastic container.

3.4.2 Extraction.

The ground leaf powder was extracted with dilute hydrochloric acid via the decoction method. The resulting extracts were basified with ammonia filtered then dried. The leaves extracts were dried and subsequently weighed to yield an extract of 31.8 g. On the crude extract was performed liquid-liquid extraction in DMSO (dimethyl sulfoxide). Extracts were then stored in a plastic container at room temperature.

3.4.2.1 Preliminary extraction.

This was done as a preliminary experiment to determine the feasibility of the extraction. A small amount of (10.0 g) the ground leaves was soaked in 100 mL of 0.01M HCl. The mixture was simmered for two hours then filtered using filter paper No. 1.To the obtained residue was added 100 mL of 0.01 M HCl and the mixture re-simmered for two hours then filtered. The resulting residue was re-simmered for another two hours after 100 mL

of 0.01 M HCl was added onto it and filtered. The filtrates 1, 2 and 3 were combined to give a clear solution. Onto the resulting solution (75 mL of 2 X 10^{-9} M) NH_4OH was added drop wise until a precipitate was formed. The mixture was then filtered and the precipitate allowed drying at room temperature.

3.4.2.2 Large-scale extraction.

A mixture of 237.6 g of leaves and 600 mL of 0.01 M HCl was simmered for two hours after which it was filtered. Onto the residue was added 600 mL of 0.01 M HCl the mixture re-simmered for two hours then filtered. The residue in step 2 above was re-simmered for another two hours after 600 mL of 0.01 M HCl had been added onto it. The mixture was then filtered. The three filtrates were combined to form a clear solution. Onto the solution was added 400 mL of 2 X10^{-9} M NH_4OH at intervals of 20 mL while shaking the flask until the precipitate would form. The mixture was then filtered and the residue dried and weighed to yield a crude extract of 31.8 g. The percentage yield of the alkaloid extracted being 13.45.

3.4.3 Wagners' test.

2 mg of the ethanolic extract was acidified with 1.5 % v/v of hydrochloric acid and a few drops of wagner reagent was added

3.4.4 Spectroscopic analyses.

The water and DMSO extracts of the leaf were analyzed, by IR NMR and mass spectroscopic techniques.

3.4.4.1 IR spectra.

The IR spectra of the samples were measured in an automatic recording IR spectrophotometer Shimadzu model-408 in the solid state mixed with potassium bromide. A thin disc was prepared under anhydrous conditions from a powder containing1mg of medicinal material and 50 mg KBr using a mould and press.

3.4.4.2 MS spectra.

MS measurements were taken at the Department of Chemistry, Faculty of Engineering and Physical Sciences, University of Surrey, Guildford, UK using a Bruker Autoflex MALDI-TOF MS via Electrospray ionization (ESI) technique.

3.4.4.3 NMR Spectra.

The 1D (^{1}H NMR) and 2D (COSY, HSQC DEPT and HMBC) NMR spectra were recorded in DMSO or D_2O on a 500 MHz Bruker AVANCE NMR instrument at room temperature at the University of Surrey (Guildford, United Kingdom). Chemical shifts, δ, were expressed in ppm and referenced against the solvent resonances at 2.50 and 39.51 ppm for ^{1}H and ^{13}C NMR respectively for deuterated DMSO and 4.80 for ^{1}H NMR for D_2O.

3.4.5 Drop pill formulation.

Formulation of drop pills was done by the dropping preparation method. This method involves circulating water of 80-85 ^{0}C supplied by the constant temperature circulator through the jacket of the liquid reservoir. The cooling column was filled with liquid paraffin of which its outside was cold water with crashed ice in it. The extract dispersion was made by placing an evaporating dish on a hot water–bath, putting 21.0 g of PEG-

6000 and stirring continuously until it was thoroughly melt .To the matrix was added 4.5 g of the dry leaf extract.

The circulation of 80-85 ^{0}C water was turned on to keep warm. The PEG-6000 with the dried leaf extract was transferred into the liquid reservoir. The distance between the dropper and the surface of the cooling agent was adjusted to 5 cm. The internal and external diameters of the burette were 2.0 and 2.4 mm respectively. The drug was dropped into liquid paraffin of 15 ^{0}C. The dropping rate of 30 drops per minute was controlled. After the drops were cooled thoroughly the pills were taken out, placed onto a filter paper, the liquid paraffin cleaned off their surface and they were dried at room conditions. The total number of pills from the 4.5 g of medicament was 20. The dried pills were then stored in a plastic bottle.

3.4.5.1 The weight variation test.

20 drop pills of the sample were used. The 20 whole drop pills were weighed precisely and an average calculated. From the average, each of the 20 drop pills were precisely compared and checked that the weight variation complied with the specifications given by Gao, (2005). The weights of not more than two drop pills should be such that they differ from the weight by the percentage listed, no drop pill differs by more than double that percentage.

3.4.5.2 Solubility test of the drop pills.

20 of the formulated pills were dissolved in water at 37 ^{0}C and the time taken to completely dissolve noted. The dissolution was repeated three times. Another set of 20 drop pills were dissolved in water at 100 ^{0}C and the time taken to completely dissolve

was also noted. The experiment was also repeated three times and the results tabulated.

3.4.6 Mosquito larvicidal assay.

Larvicidal effect of 2nd, 3rd and 4th in star larvae of mosquito were investigated in a dose – dependent manner for a period of 48 hrs. The larvae were exposed to test concentrations of 0.5, 0.1 and 0.15 mg /mL of DMSO extract in the laboratory at $26 \pm 1\ ^{0}C$ while being provided with dry yeast powder. 100 mL of water was taken in a series of 250 mL glass beakers. The measured amount of extracts was dissolved in 1mL of DMSO (the solvent which was used for preparing the extract). The dissolved leaf extract was added to the water in the beakers. A control was maintained by adding 1mL of DMSO to 100 mL of water. 20 Larvae per concentration were used for all the experiments. The larvae were fed on dry yeast powder on the water surface. The number of dead larvae at the end of every 24 h was recorded and the mortality percentage values were calculated. This experiment was repeated three times and the results were recorded in a table.

3.4.7 Antifungal assay.

Leaves of *M. foetida* were screened for their antifungal potential using the disk diffusion method. The powdered leaves and crude extracts were screened against the fungi pathogen *C. albicans.*

3.4.7.1 Test microorganisms.

The microorganisms were obtained from the Moi Teaching and Referral Hospitals' Microbiology laboratory then stored in a refrigerator. Test microorganisms consisted of the fungal strain *C. albicans*.

3.4.7.2 Extract solution.

The extract solution was made up of 0.035 g of dried extract of *M. foetida* weighed and transferred into a 10 mL volumetric flask. Distilled water was then added to make up the solution (0.035 g in 0.01 L).

3.4.7.3 Agar Preparation.

Potato Dextrose Agar was used to make up the medium for fungi. Potato was peeled and 100 g was measured finely chopped and boiled to a mash in distilled water. Dextrose was measured (12.5 g) and placed in a 1L measuring cylinder. Agar was measured (12.5 g) and added to the measuring cylinder. Hot distilled water was added to make up 500 mL. The contents were continuously poured and stirred until consistency was achieved. The content was then poured into a conical flask plugged with cotton wool over which a foil was tightly wrapped. The flask was then autoclaved at 121 ^{0}C for 24 h. The pH range was maintained at 6.5-7.0. Nystatin was used as a reference drug. The control experiment included a plate of solidifying agar onto which was inoculated pure distilled water with fungi mixed in a 1:1 portion.

3.4.7.4 Preparation of inoculums.

The Petri plates were washed thoroughly and sterilized in hot air oven at 160 ^{0}C for 90 minutes. 30 mL of sterile dextrose agar medium seeded by organisms in semi hot conditions was poured aseptically in sterile Petri plate and allowed to solidify at room temperature. Bores were made on medium using sterile borer and 0.1 ml of the standard at a concentration 0.5, 1.0 and 1.5 mg/ mL. The Petri plates were incubated at 29-31 ^{0}C for 48 h in a BOD incubator and zone of inhibition was observed and measured using a scale. All the tests were performed in triplicate.

3.4.7.5 Disk diffusion method.

An inoculum containing yeast cells was applied onto Sabaroud agar plates. On each plate a reference antibiotic was also applied. Reference antibiotic contained 0.5, 1.0 and 1.5 mg of antibiotic per mL The disks were made by cutting discs 5.6 mm from a filter paper with a perforator placing 3 of these discs in a vial and adding 0.2 mL of each extract solution. These were left to dry. Discs were also made for the controls. Each disc was impregnated with the *M. foetida* leaf extract at appropriate concentration of 0.5, 1.0 and 1.5 mg/ mL using a μL syringe. This was then incubated with the test organism-fungi *C.albicans*. Incubation was done at 37 ^{0}C for 48 h. Discs were applied to the plates already streaked with the fungus.

3.4.7.6 Determination of zone of Inhibition.

The leaf extract was dissolved in DMSO to get the concentration of 0.5, 1.0 and 1.5 mg/mL. Evaluation of the activity was carried out by cup-plate technique using dextrose agar medium for fungi strain.

CHAPTER FOUR

RESULTS AND DISCUSSION

In this study a brown and shiny solid (the alkaloid) was confirmed by a positive Wagner's test. Infrared analysis was used to confirm the functional groups in the alkaloids.

4.1.1 IR Spectra.

The infrared spectra of both the water and the DMSO leaf extracts were done to determine the functional groups present in them. Table 4.1 shows that there was a broad peak at 3200 cm^{-1}, a relatively narrow peak at 1640 cm^{-1} and a sharp peak at 1380 cm^{-1}. The IR Spectra of all the samples showed a strong band in the region 3500-3100 cm^{-1} indicating the presence of secondary amine and/or hydroxyl functional groups in the samples (Kemp 1991).

A medium intensity band to the right of the C=O absorption was probably caused by the N-H bending vibration. In solid phase this vibration often overlap the C=O absorption. The N-H bend in secondary amines is observed near 1500 cm^{-1}(Kemp, 1991).

The C-N stretch occurs in the range from 1350-1250 cm^{-1} as medium to strong band for aromatic amines. The C-N absorption occurs at a higher frequency in aromatic amines because resonance increases the double bond character between the ring and the attached nitrogen atom. The bands are shifted to the left because the hydrogen bonding between the amine and the DMSO molecules reduces the frequency in the solid state (Kemp, 1991).

Table 4.1 IR Spectra of the water leaf extract.

Functional group	N-H, O-H	C=O	C-H bend	C-N
Frequency range(cm^{-1})	3450-3100	1640-1600	1400-1380	1320-1300

Table 4.2 IR Spectra of the DMSO filtrate of the leaf.

Functional group	N-H, O-H	C=O	C=C	C-H bend
Frequency range (cm^{-1})	3400-3150	1640-1615	1405-1395	1025-1010

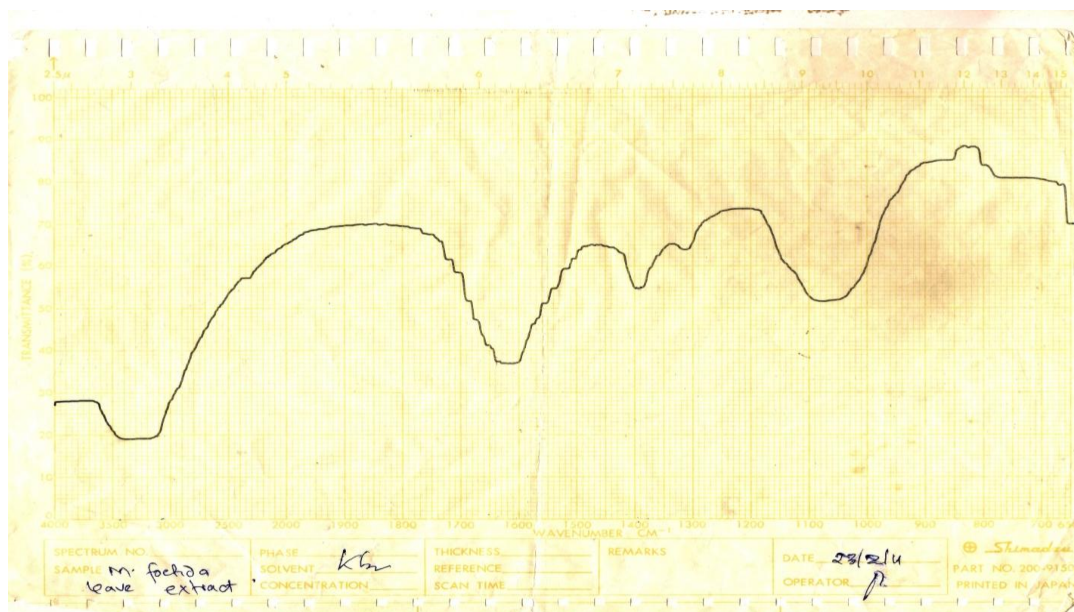


Figure 4.1 IR Spectra of the water leaf extract.

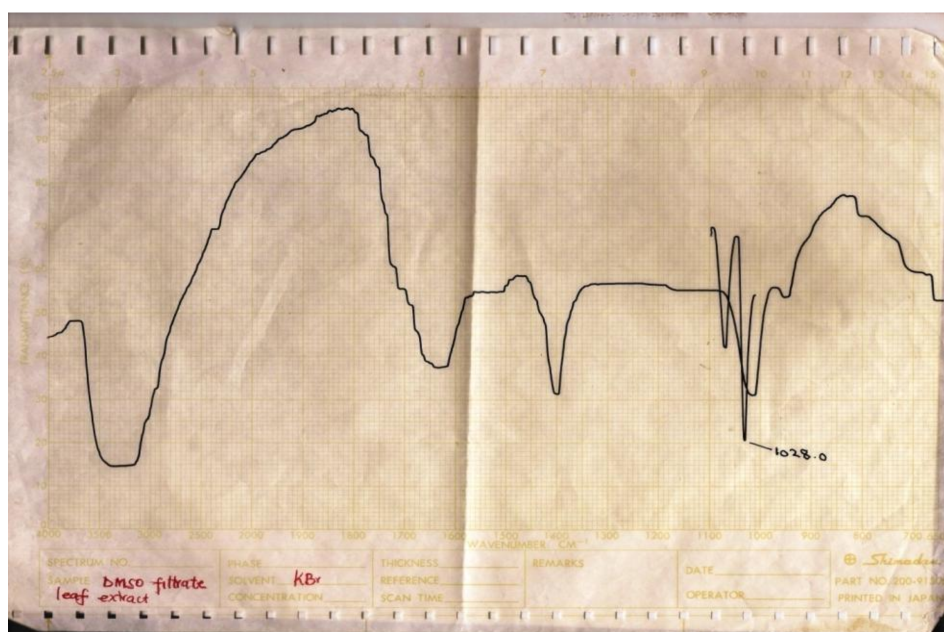


Figure 4.2 IR Spectra of DMSO leaf filtrate.

4.1.2 NMR and MS measurements

NMR and MS measurements for the DMSO and water leaf extract of *M. foetida* were recorded.

4.1.2.1 NMR and MS measurements for the DMSO leaf extract of M. foetida

1D (^{1}H NMR) and 2D (COSY, HMBC and HSQC DEPT) measurements were used to analyze the DMSO and water leaf extract of *M. foetida*. The ^{1}H NMR spectrum of the DMSO extract shown in Figure 4.1 suggest aromatic proton resonances at δ 7.80 d (J = 9 Hz), 7.47 s, 7.37 s, 7.27 s and 6.84 d (J = 9 Hz). The coupling constants for the two doublet proton resonances at δ 7.80 and 6.84 was the same at J= 9 Hz indicating that they were coupled. This coupling was confirmed by a correlation observed in COSY spectrum (Figure 4.2). The magnitude of the coupling constant suggested that the double bond splitting system was either due to the *ortho* coupling in an aromatic system or *cis*– double bond that neighbors a carbonyl system. This was previously observed by Paiva, 2013. A pair of coupled doublet proton resonances that could be attributed to either oxygenation of double bonds at δ 4.89 d (J = 11.3 Hz) and 4.69 d (J = 11.3 Hz) and a proton resonance at the shoulder of HOD peak of the deuterated solvent at δ 3.49 m suggesting the presence of a hydroxyl group were also observed in figure 4.2.

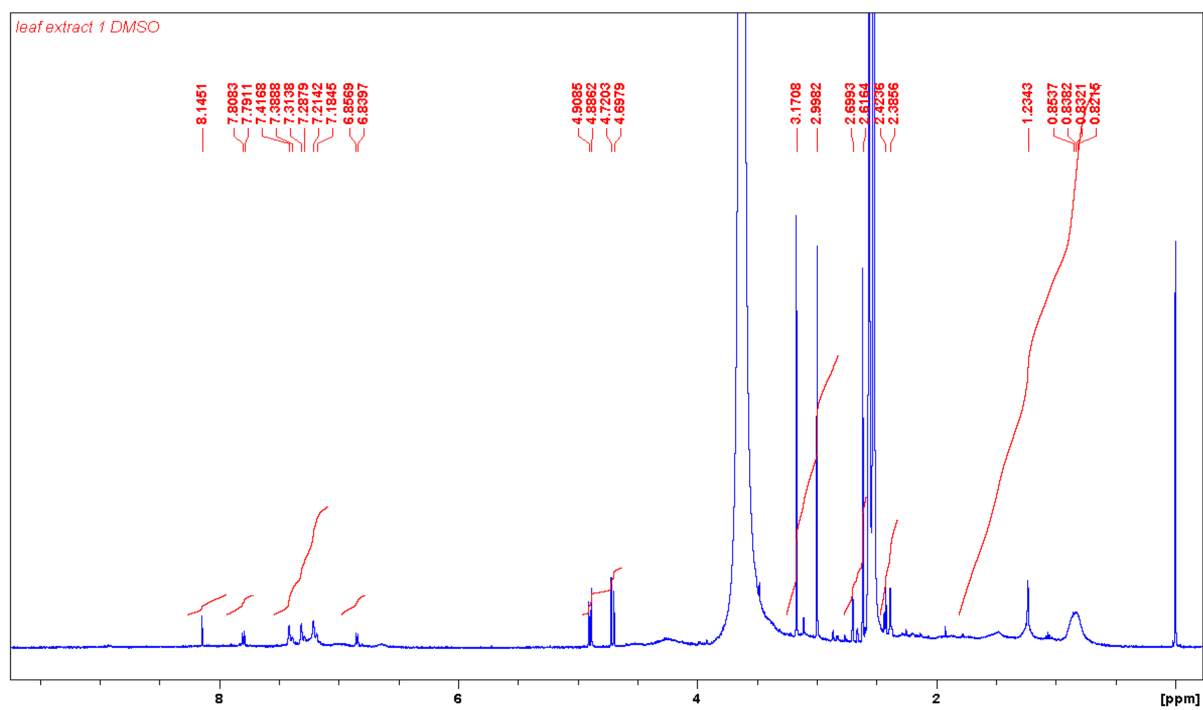


Figure 4.3 ^{1}H NMR spectrum for DMSO leaf extract of *M. foetida.*

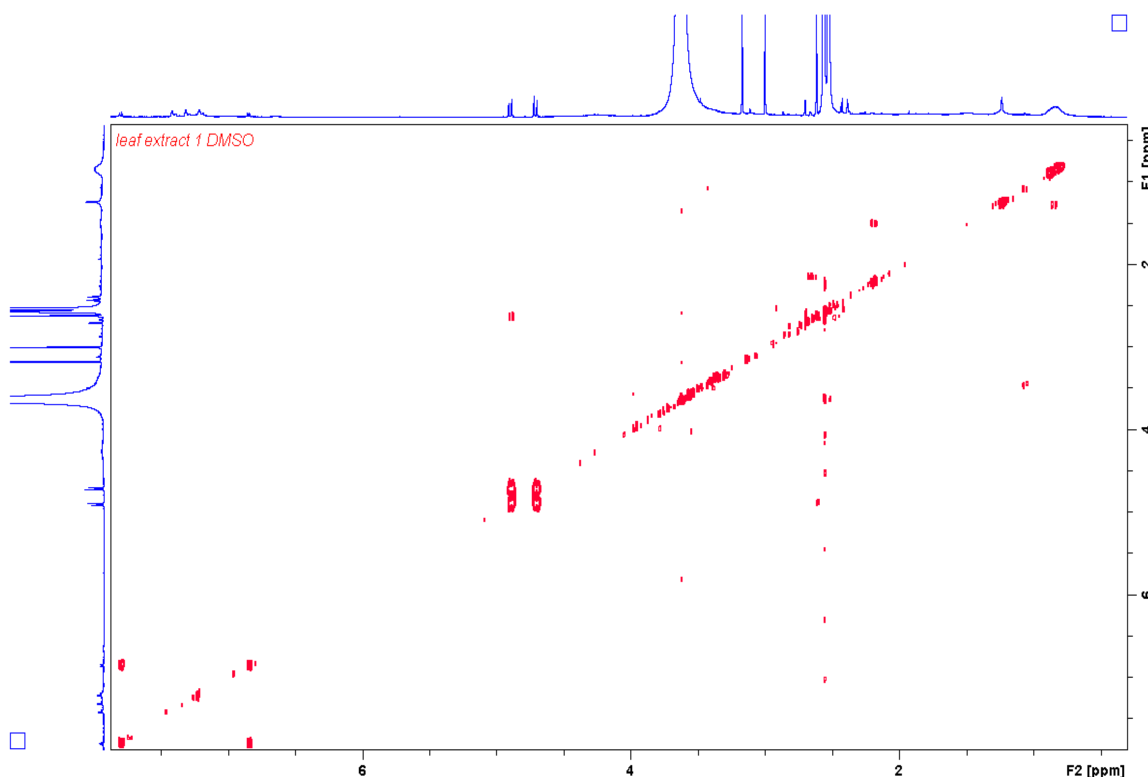


Figure 4.4 COSY NMR spectrum for DMSO leaf extract of *M. foetida*.

Interestingly three characteristic methyl group proton resonances were observed at δ3.17 s, 3.00 s and 2.62 s. The chemical shifts of these methyl groups suggesting that they were methyl groups attached to a nitrogen group of an alkaloid as observed by Paiva, 2013. In addition methylene and methine proton resonances were also observed between 0.5 – 2.69 ppm at δ 2.69, 2.42, 2.39, 1.25 and 0.83 ppm. Apart from the observed chemical shifts and multiplicities, integral lines (marked in red) were distinctive for this compound(s) showing a reasonable ratio of 1:2:3 for clear methine, methylene and methyl group resonances (Figure 4.2). This finding suggested that this compound was pure and the above ^{1}H and COSY NMR spectroscopic data suggested that the compound present in the DMSO leaf extract of *M. foetida* was an aromatic alkaloid possessing three N-CH_3 groups, hydroxyl groups and a saturated moiety.

As the quantity of the sample submitted for spectroscopic analysis was in low yield ^{13}C and DEPT NMR measurements could not be determined. However, 2D NMR measurements capable of providing information on the types of carbons present were conducted. The 2D NMR procedures were HSQC DEPT and HMBC that gave information on correlation between proton and carbons. THE HSQC DEPT spectrum showed a correlation of a proton resonance and a carbon that is attached to it whereas the HMBC spectrum showed a correlation between a proton and carbon resonances that either 2 or 3-bonds away from it. From the HSQC DEPT spectrum (Figure 4.3) and HMBC spectrum (Figure 4.4) the functional groups observed in the ^{1}H NMR measurement were confirmed. From the above NMR observed data the compound present in the DMSO leaf extract of *M. foetida* was most likely a derivative of zeatin riboside reported previously from the genus *Momordica* (Dictionary of Natural products, 2014). The infrared spectrum of the DMSO leaf extract of *M. foetida* also confirmed presence of hydroxyl, NH, CH, carbonyl and double bonds in an alkaloid derivative of zeatin riboside.

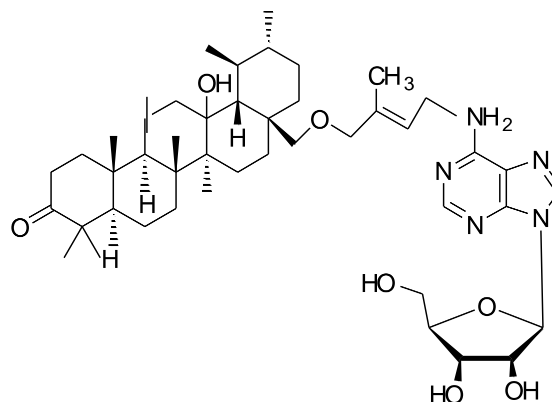


Figure 4.5 Proposed structure of the alkaloidal derivative of zeatin riboside.

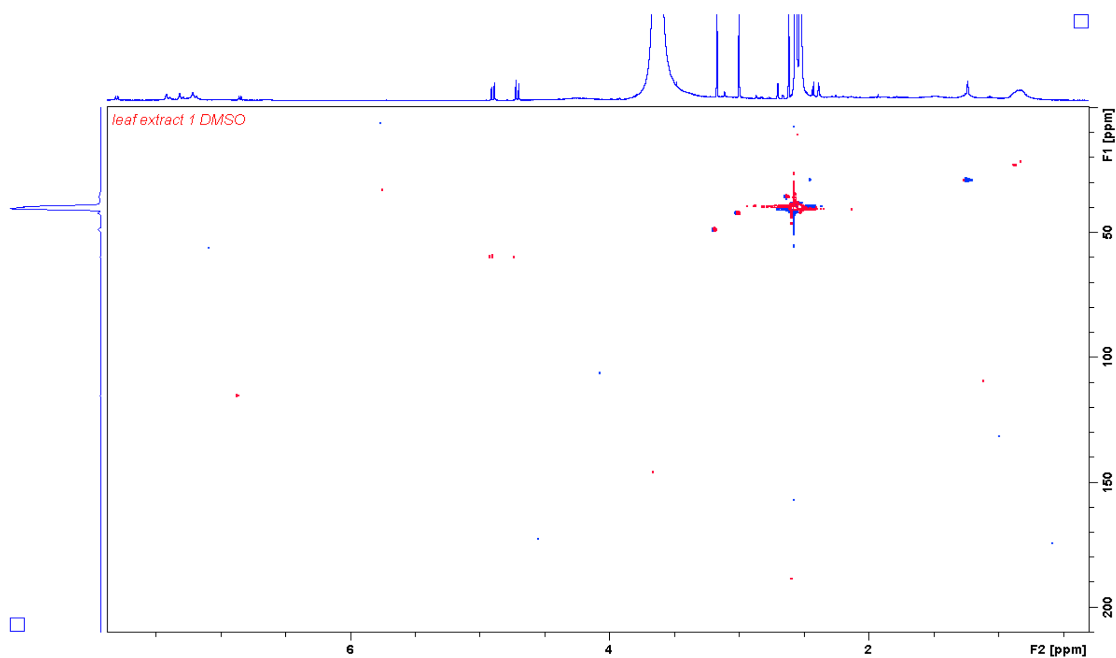


Figure 4.6 HSQCDEPT NMR spectrum for DMSO leaf extract of *M. foetida*.

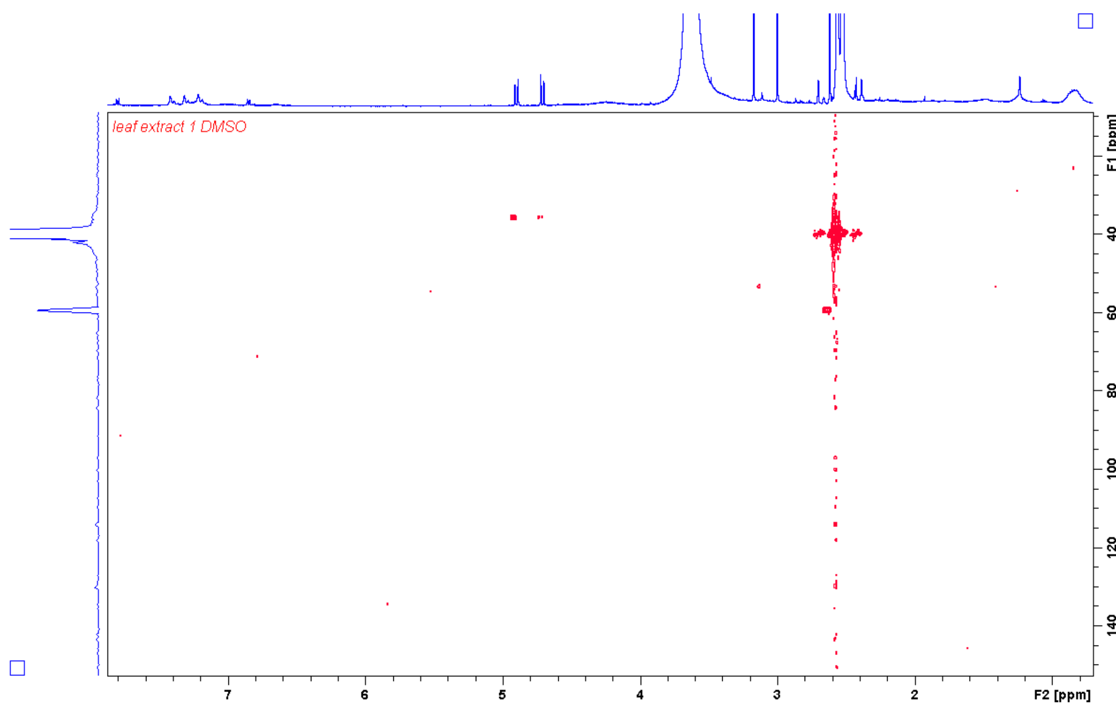


Figure 4.7 HMBC NMR spectrum for DMSO leaf extract of *M. foetida*.

The MS procedure (Figure 4.5) was also used to confirm the molecular formula proposed for the structure. A molecular ion peak at *m/z* 645 being an odd value suggested that the alkaloid has an odd number of nitrogens. Fragment ions were observed at 613, 596, 555, 515, 469, 426, 339, 323, 296 and a molecular base peak at 255.

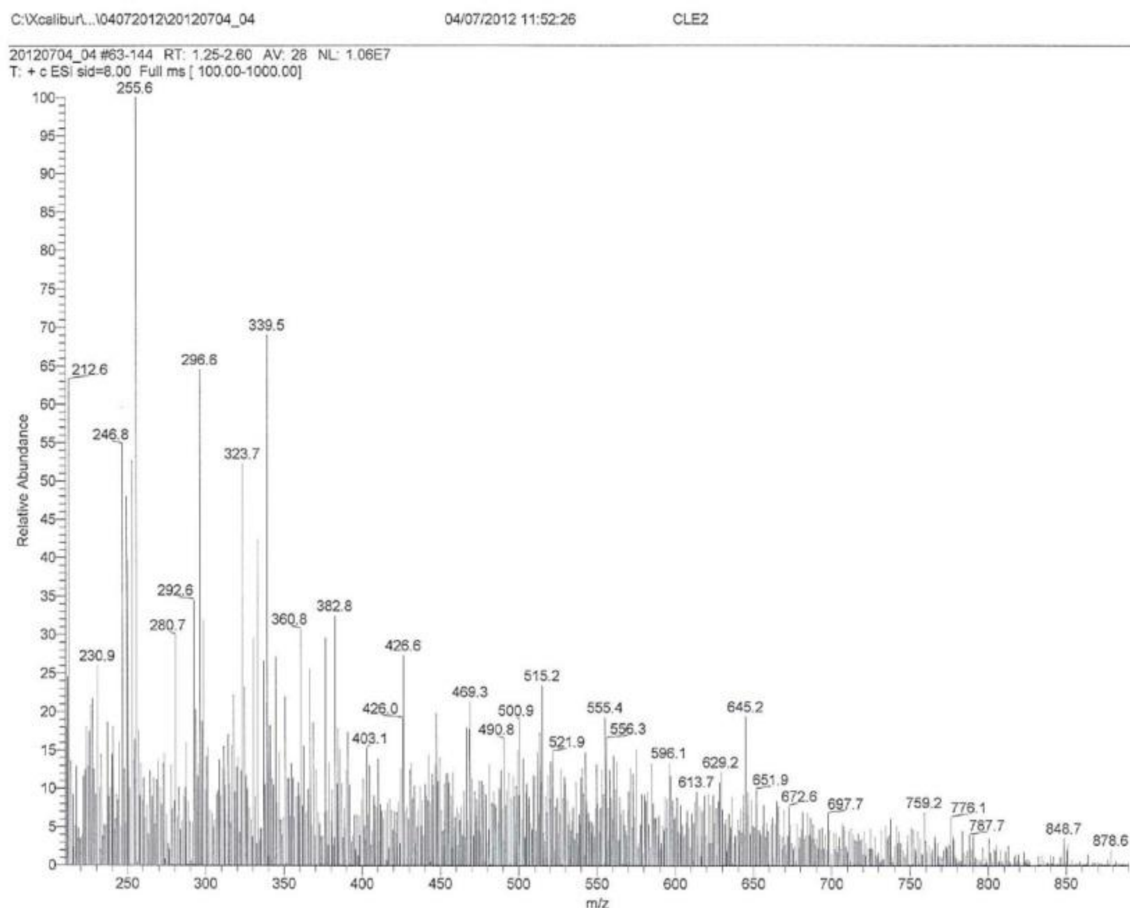


Figure 4.8 MS spectrum for DMSO leaf extract of *M. foetida.*

4.1.2.2 NMR and MS measurements for the water leaf extract of *M. foetida*

The ^{1}H NMR spectrum of the water leaf extract of *M. foetida* is shown in Figure 4.6. The observed proton resonances at δ 7.47 (s, aromatic-CH), 7.37 (s, aromatic-CH), 7.27 (s, aromatic-CH), 3.17 (s, CH_3) and 3.00 (s, CH_3) which were also observed in the DMSO

leaf extract of *M. foetida*. The resonances were consistent with N-CH_3 and aromaticity observed for the DMSO leaf extract of *M. foetida*. The compound present in the water leaf extract was therefore determined to be closely related to the compound present in the DMSO leaf extract. The MS spectrum for this extract also showed a molecular ion peak at *m/z* 596 and other fragments at 580, 556, 512, 466, 382 and a molecular base peak at 336 (Figure 4.7). The compound present in the water extract was also determined to be an alkaloid derivative of zeatin riboside.

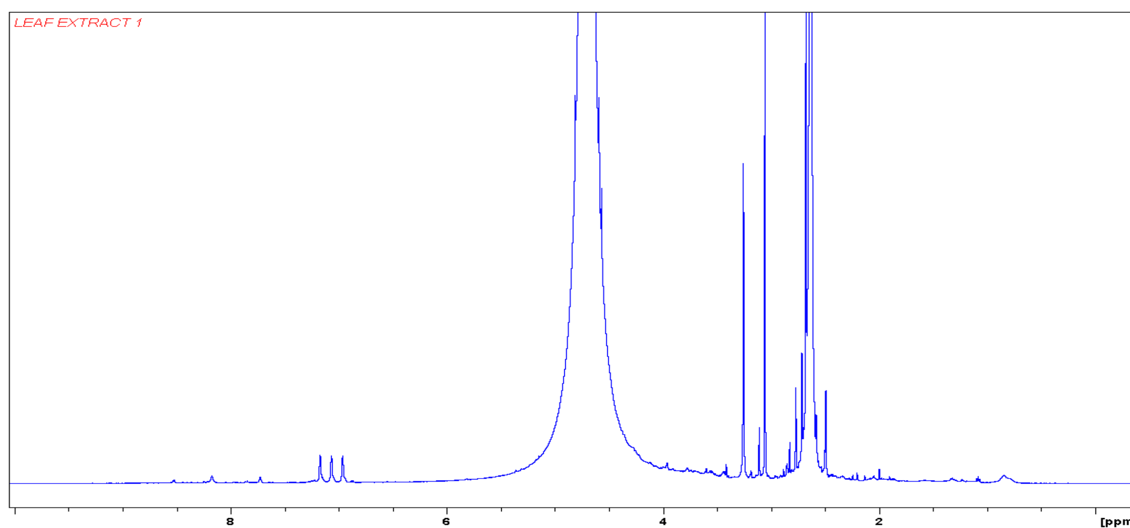


Figure 4.9 ^{1}H NMR spectrum for crude water leaf extract of *M. foetida*.

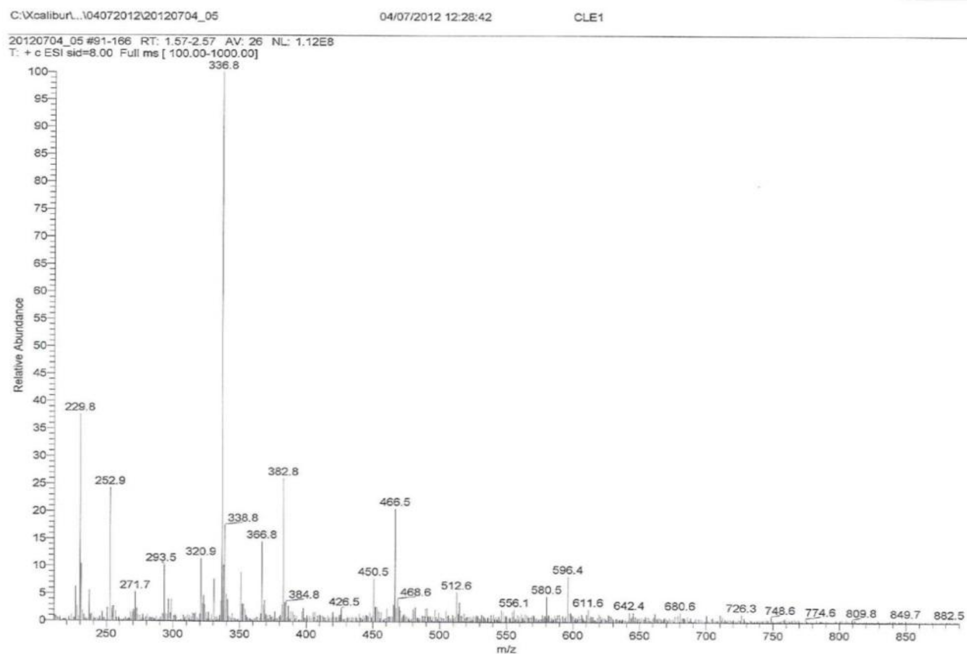


Figure 4.10: MS spectrum for water leaf extract of *M. foetida.*

4.2 Drop pill formulation

The formulation of drop pills resulted in the formation of pills that are shown in Plate 4.1**.**

Plate 4.1 A photograph of the formulated drop pills (Source: Author, 2010).

4.2.1 Quality control aspects

The pills were evaluated on the basis of size, weight, color and solubility in water.

4.2.2.1 Appearance.

The size of one pill was found to be approximately 0.03001g. The observed variation in the size and color of the pills was due to the difference in the dripping time owing to the large dropper that was used in the formulation. The pills which appear lightly colored took a longer time to drip into liquid paraffin. This might have been due to variations in the amounts of excepients added.

4.2.2.2 The weight variation test.

Twenty drop pills were picked at random and the weight of each determined and recorded as shown in Appendix 1. The average weight of the twenty pills was then calculated and the weight compared with that recommended by Gao (2005).

According to Gao (2005) the weight variation should conform to Table 4.2. From the study, it was found that the weight of each pill was within the range of 0.0276g and 0.0305g which falls within the ±15% variation range described by Gao (2005). The average weight of 20 pills was 0.0297 grams.

The average weight =

(0.0297+0.0301+0.0299+0.0305+0.0301+0.0289+0.0304+0.0299+0.0287+0.0291+0.030 0+0.0299+0.0289+0.0305+0.0301+0.0305+0.0299+0.0276+0.0293+0.0301) /20 = 0.0297 grams

Table 4.3 Weight variation test (Gao 2005):

Average weight of drop pills in sample	% deviation about the average weight
0.03g or less	±15
From 0.03 through 0.1g	±12
From 0.1g through 0.3g	±10
More than 0.3g	±7.5

4.2.2.3 Solubility in water.

When the formulated pills were dissolved in water at 37 ^{0}C and 100 ^{0}C respectively their dissolution rates were determined, by averaging the time taken for all pills to dissolve as shown in Table 4.4. The formulated pills were found to dissolve in 6.9 minutes in heated water to a temperature of 100 ^{0}C which is within the 10 minutes allowed dissolution time as per the requirements described by Gao (2005). When 6 drop pills were dissolved in water warmed to a temperature of 37 ^{0}C, they dissolved in 22 minutes which is also within the range proposed by Gao (2005) of 30 minutes. This solubility of the drop pills showed that the release rate of the pills is high enough.

Table 4.4 A comparison of the amount of time taken for the drop pills to dissolve in water at 37^0C and 100^0C.

Number of occasions the pills were dissolved	Time taken for all the pills to dissolve in water at $37\ ^0$C	Time taken for all the pills to dissolve in water at $100\ ^0$C
1	22.0	6.8
2	22.1	7.1
3	21.9	6.9

Average time = (22.0 + 22.1+21.9) /3 =22.0 minutes

Average time = (6.8 +7.1+ 6.9) /3 = 6.9 minutes

4.3 Bioassays.

Bioassays on the extracts involved mosquito larvicidal assay and antifungal assay on *Candida albicans.*

4.3.1 Mosquitocidal assay.

Bioassays with the DMSO extract of *M. foetida* against larvae of *Ae. aegypti* revealed on average the % mortality values of 68, 63 and 77 for the 2nd in star larvae as shown in list of appendices for 0.05,0.10 and 0.15 mg /m L of the DMSO extract.

The % mortality values of 22, 63 and 80 (appendices list) for the 3rd in star larvae and 22, 48 and 80 for the 4th in star larvae (appendices list) for the respective concentrations. Results of the experiment conducted for evaluating the larvicidal efficacy of the extract showed that they are toxic to mosquito larvae. Lethal concentrations of the leaf extracts

were 0.15 mg /mL for the 3rd and 4th in star larvae (appendices list) and 0.05 mg /mL for the 2nd in star larvae (appendices list) after 24 h of exposure. When the 2nd, 3rd and 4th in star larvae were treated with 0.05, 0.10 and 0.15 mg /mL of Supa kill which was also used as a reference in this study all the larvae died within 30 minutes of continous exposure (appendices list). *M. foetida* has shown good larvicidal activity against *A.aegypti* mosquitoes in laboratory settings. Mosquitoes in the larval stage are attractive targets for pesticides because they breed in water and they are easy to deal with them in this habitat. The 24h bioassay is a major tool for evaluating the toxicity of phytotoxins and researchers have been applying this method to assess the toxic effect of different plant extracts against mosquitoes (Singh *et al*., 2006).

Table 4.5 DMSO leaf extracts mortality effect.

Larvae / conc (mg /mL)	2nd in star	3rd in star	4th in star
0.05	68	22	22
0.10	63	63	48
0.15	72	80	80

Table 4.6 The mortality effect of Supa Kill (Synthetic pyrethrin) on mosquito larvae.

Amount in mL	Concentration in mg / mL	Time taken for all larvae to die (min)	Average no. of dead larvae	% mortality
Control	0	0	0	0
2.8	0.05	30	20	100
5.6	0.10	30	20	100
8.5	0.15	30	20	100

4.3.2 Antifungal assay.

Results show that the zone of inhibition diameter of the water and DMSO extracts against *Candida albicans* is on average 0.4 mm (Table 4.7) and 0.0 mm (Table 4.8) respectively. This means that *Candida albicans* is sensitive to the water extract since it is inhibited with a diameter of 0.4mm but is resistant to the DMSO extract since it grows to the edge of the disk. The magnitude of the zone of inhibition diameter, 0.4 mm, obtained in this experiment also shows that the antifungal activity of the water extract is low. This value is even less than that of nystatin which was used as a reference drug in this experiment (whose ZID is 1 mm).

Table 4.7 Zone of Inhibition diameters of the water extract.

Concentration of crude extract in mg/mL	0.5	1.0	1.5
Zone of inhibition diameter (ZID) (mm)	0.39	0.40	0.41

Table 4.8 Zone of inhibition diameters of the DMSO extract.

Concentration of DMSO extract in mg/mL	0.5	1.0	1.5
Zone of inhibition diameter (ZID) (mm	0.0	0.0	0.0

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The study has been able to successfully characterize the active compound in *M. foetida* leaf extract as an alkaloidal derivative of zeatin riboside.

This study has also been able to demonstrate significant larvicidal activity of *M. foetida* which could be used in insecticide formulation. The results in the larvicidal assay are high with the maximum larvicidal activity being observed at 0.15 mg / mL on the 3rd and 4th in star larvae. Toxicological studies have shown that *M. foetida is* safe for human health and there is no toxic effect since *M. foetida* is used as a vegetable for human consumption. Hence the larvicidal action of the leaf extract of *M. foetida* could be exploited for use in potable waters against mosquito larvae.

The antifungal activity of the water extract was lower than that of nystatin, a standard antibiotic; this was also used as a reference in this study. Drop pills of the extract that enable effective dosage administration were successfully formulated.

5.2 Recommendation

In vivo studies on the plant should be done to determine its efficacy and mechanisms of action. Since the % yield of the alkaloid in the leaf is low (13% in this study) the plant should be cultivated for large scale application. A dropper of a smaller diameter (< 2mm) should be employed for the formulation of well rounded drop pills.*M. foetida* could be exploited to effectively manage mosquitoes especially in stagnant waters around the home.

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APPENDICES

Appendix I: Weight of 20 drop pills.

Number	Weight (grams)	Number	Weight (grams)
1	0.0297	11	0.0300
2	0.0301	12	0.0299
3	0.0299	13	0.0289
4	0.0305	14	0.0305
5	0.0301	15	0.0301
6	0.0289	16	0.0305
7	0.0304	17	0.0299
8	0.0299	18	0.0276
9	0.0287	19	0.0293
10	0.0291	20	0.0301

Appendix II: DMSO leaf extract mortality effect on 2nd in star larvae.

Concentration in mg/mL	Total no. of larvae	No. of larvae	% mortality
Control	20	0	0
0.05	20	12	60
0.10	20	13	65
0.15	20	15	75

Appendix III: DMSO leaf extracts mortality effect on 2nd in star larvae.

Concentration in mg/mL	Total no. of larvae	No. of larvae	% mortality
Control	20	0	0
0.05	20	14	70
0.10	20	13	65
0.15	20	15	75

Appendix IV: DMSO leaf extract mortality effect on the 3rd in star larvae.

Concentration in mg/mL	Total no. of larvae	No. of larvae	% mortality
Control	20	0	0
0.05	20	4	20
0.10	20	12	60
0.15	20	15	75

Appendix V: DMSO leaf extracts mortality effect on the 3rd in star larvae.

Concentration in mg/mL	Total no. of larvae	No. of larvae	% mortality
Control	20	0	0
0.05	20	4	20
0.10	20	11	55
0.15	20	16	80

Appendix VI: DMSO leaf extracts mortality effect on the 3rd in star larvae.

Concentration in mg/mL	Total no. of larvae	No. of larvae	% mortality
Control	20	0	0
0.05	20	5	25
0.10	20	12	60
0.15	20	17	85

Appendix VII: DMSO leaf extract mortality effect on the 4th instar larvae.

Concentration in mg/mL	Total no. of larvae	No. of larvae	% mortality
Control	20	0	0
0.05	20	4	20
0.10	20	10	50
0.15	20	17	85

Appendix VIII: DMSO leaf extract mortality effect on the 4th instar larvae.

Concentration in mg/mL	Total no. of larvae	No. of larvae	% mortality
Control	20	0	0
0.05	20	5	25
0.10	20	12	60
0.15	20	16	80

Appendix IX: The mortality effect of DMSO leaf extract on the 4th instar larvae.

Concentration in mg/mL	Total no. of larvae	No. of larvae	% mortality
Control	20	0	0
0.05	20	4	20
0.10	20	11	55
0.15	20	15	75

Appendix X: The mortality effect of Supa Kill (Synthetic pyrethrin) on mosquito larvae.

Amount in mL	Concentration in mg/mL	Average no. of larvae	% mortality
Control	0	0	0
2.8	0.05	20	100
5.6	0.10	20	100
8.5	0.15	20	100