

**TAXONOMIC STUDIES OF *Lippia javanica* (BURM F.) SPRENG. (VERBENACEAE)
COMPLEX IN EAST AFRICA USING MORPHOLOGICAL AND MOLECULAR
CHARACTERS AND EVALUATION OF ITS PESTICIDAL EFFICACY ON APHIDS**

BY

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AND ECONOMIC BOTANY**

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Declaration

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Dedication

This work is dedicated to my parents Mrs. Maryline Nakhungu Mutonyi and the late Mr. Dickson Wanyama Masinde for their love upon me.

Abstract

Lippia javanica (Burm. F.) Spreng occurs naturally in eastern, central and southern Africa. It has widely been used as herbal tea, for ethnomedicinal applications and as a botanical pesticide. *Lippia javanica* species exhibit great morphological and chemical variations which are notable across a number of natural populations. Differences in morphological taxonomy on important characters such as peduncle length and number of inflorescence per axil have been reported which has led to the recognition and identification of infraspecific taxa of var. *whytei* auct and var. *javanica* in Southern Africa region, creating a major taxonomic conflict in *Lippia javanica*, a scenario which needs to be resolved. Many plant extracts show a broad spectrum of activity against pests and such products have long been touted as attractive alternatives to synthetic chemical pesticides for pest management because they pose little threat to the environment. There is a general paucity of information on taxonomic studies of *L. javanica* on the morphological, molecular and chemical properties, which can aid in the comprehensive understanding of the taxonomy and classification of *L. javanica* in East Africa region. Chemotaxonomic studies have shown strong chemical variations in phytochemical compounds of *L. javanica* in other African regions with little information available in Kenya. There is hardly any information on the efficacy of the extracts of *L. javanica* on common aphids of cowpeas in Kenya. The purpose of this study was to undertake taxonomic studies on the proposed variants var. *javanica* and var. *whytei*. of *L. javanica* complex based on morphometrics, molecular and phytochemical characters, and evaluate its efficacy as a botanical pesticide for control of aphids (*Aphis craccivora*) (C.L) Koch of cowpeas (*Vigna unguiculata*). Data on morphological characters were obtained from the national Museums of Kenya herbarium preserved specimens. Observations were also made from specimens collected from Maralal, Naivasha, Nyahururu (Rumuruti) Sotik, Sondu, Kedong, Kapsowar, Ngong and Narok sites of Kenya to integrate this information with the morphological data from the herbarium preserved specimens. Molecular studies involved the use of Inter-simple sequence repeats and Random Amplified Polymorphic Deoxyribonucleic acid to determine genetic variation of freshly collected young leaf tissues. The collected field specimens were screened to establish their phytochemical composition. A completely randomized block design with five treatments (1%, 5%, 10%, positive, and negative controls) was used to evaluate the efficacy of *L. javanica* extracts against the aphids in the field. Cluster analysis and principal component analysis of morphological data revealed that *L. javanica* had no significant variations. Similar band pattern were also portrayed by all the analyzed specimens using Inter Simple Sequence Repeats and Random Amplified Polymorphic Deoxyribonucleic acid markers. The morphological and molecular study revealed that *L. javanica* is one species. The species was found to be rich in a variety of phytochemical compounds such as phenols, flavonoids, tannins, alkaloids, and terpenoids that perhaps exhibited pesticidal effects on the aphids. Phenolic glycosides, resins and polynuroids were absent. Application of the various concentrations of *L. javanica* extracts significantly suppressed the abundance of aphids on cowpeas at $P \leq 0.05$. The mixture of *L. javanica* extracts at 10% was more effective than 1% and 5% extracts concentration. The 10% extract was not significantly different from the synthetic pesticide hence can be a suitable botanical pesticide for controlling *Aphis craccivora* of cowpeas. The findings from this study would greatly assist taxonomists and plant systematists in correctly classifying and identifying the highly variable *Lippia javanica*, and will be useful to farmers in the control of common aphids of cowpeas.

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List of Abbreviations and Acronyms

AFLP - Amplified Fragment Length Polymorphism

ANOVA - Analysis of the Variance

ASL-Above Sea Level

CA-Cluster Analysis

CONC.-Concentration

DA-Discriminant Analysis

DNA-Deoxyribonucleic acid

DNTPs-Deoxynucleotide triphosphate

EA-East Africa

ED-Euclidean Distance

FTEA- Floral of Tropical East Africa

FIG-Figure

G- Grams

GPS-Geographical Positioning System

HAB-Habitat

LSD-Least Significant Difference

ISSR- Inter-Simple Sequence Repeats

KALRO- Kenya Agricultural and Livestock Research Organization

K-Kenya

L-Liters

ML-Milliliters

MM-Micro Molar

NMK-National Museums of Kenya

OPTIONs-Optimization of Pesticidal Plants Innovation Outreach and Networks

OTU –Operational Taxonomic Units

PCA-Principal Component Analysis

PCR-Polymerase Chain Reaction

RAPD- Random Amplified Poymorphic Deoxyribonucleic Acid.

TAE–Tris Acetate Ethylenediaminetetra acetic acid

Tz- Tanzania

UPGMA-Unweighted Paired Group Mean with Averages

UV-Ultra Violet

Ug-Uganda

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CHAPTER 1: INTRODUCTION

1.1 Background information.

Lippia javanica (Burm F.) Spreng more often than not referred to as lemon bush is a multi-branched woody shrub (Kamanula *et al.*, 2017) of the tribe Verbenae, family Verbenaceae (Munir, 1993). It is a strongly fragrant medicinal indigenous plant that is widely spread in eastern and southern tropical Africa (Van Wyk *et al.*, 2008). The species is locally abundant in disturbed and rocky hill sites as well as grasslands, open woodlands, fringes of afro-montane forests and riverbanks. According to Kamanula *et al.* (2017), *L. javanica* is a drought resistant and can grow in a variety of soil types.

The taxonomy of the *L. javanica* is still unclear (Munir, 1993; Verdcourt, 1992; Kamanula *et al.*, 2017). Verdcourt (1992) posits that the aforementioned species possesses somewhat overlapping variation of the important characters which are the peduncle length and number of inflorescence per axil. There were even attempts to separate *L. javanica* into two varieties; variety *javanica* with long peduncles and variety *whytei* being more or less sessile; however, the characters were not constant enough (Verdcourt, 1992). Due to the variations exhibited by this medicinal shrub, there still exists a taxonomic conflict. Despite the fact that Verdcourt (1992) acknowledges existence of a taxonomic conflict, not much have been done in efforts to resolve the contradiction. As a matter of fact, Munir (1993) observes that the two “varieties” *Lippia javanica* var. *javanica* and *Lippia javanica* var. *whytei* are sometimes combined on the same herbarium sheet as they grow together in a number of populations.

The complexion of the morphological taxonomy in *Lippia javanica* is not new in East and Central Africa. *Lantana alba* Mill. and *Lippia javanica* were thought to be conspecific (Verdcourt, 1992). As a result, *Lippia alba* was placed as a synonymy of *L. Javanica* (Munir,

1993). The type specimen of *L. javanica*, now preserved in the Delessert Herbarium at Geneva, but there is no indication of him studying the type of *Lippia alba*, now preserved in the Philip Miller herbarium at the British Museum. Munir (1993) stated that after the types of the two species were examined, they were found to be discrete. Verdcourt (1992) noted that some specimens of the lemon bush from the Rift valley in Kenya e.g. Glover 2125 and Olodungoro, Entasekera and Starzenski 36 are exceptional in having long peduncles. It is recorded by Verdcourt (1992) that there were attempts to revise the two “sub-species”, var. *javanica* and var. *whytei*, one with pedunculate heads and the other with them more or less sessile. Nevertheless, the available information shows that the taxonomic studies in *Lippia javanica* have not been fully resolved a scenario which needs further study.

Singh (2016) notes that chemotaxonomy is the classification of organisms more so plants based on the chemical structure of the secondary metabolites. Thus the study of the compounds is very vital to taxonomists, phytochemists, and pharmacologists in solving taxonomical problems. Chemotaxonomic studies indicated chemotypes based on essential oils found in *Lippia javanica*; for instance, major components in the essential oils were reported to be myrcenone, myrcene and (*E*)- and (*Z*)-tagetenone (Montanari *et al.*, 2011). Other essential compounds were found to be caryophyllene, linalool, and *p*-cymene (Muzemu *et al.* 2011). Variations in the essential compounds of *L. javanica* samples from the same location in Zimbabwe; for instance, linalool which exhibited a range between 1.7 and 27% has been reported. Studies in Tanzania also found two new chemotypes which included geranial and neral. *Cis*-sabinene hydrate (20.3%) and limonene (13.9%) were shown to be the chemotypes of *L. javanica* in Ethiopia, with *Cis*-sabinene reported for the first time as a constituent of *L. javanica* leaf oil (Endris *et al.*, 2016a). However, most studies have focused on the essential oil composition of *L. javanica*

complex omitting the non-essential compounds. In Kenya, Mwangi *et al.* (1991) found out mycenone, *cis*- and *trans*- ocimenone to be the major constituents of essential oils. It is worth to note that alkaloids, amino acids, flavonoids, iridoids, triterpenes as well as several minerals have been isolated from *Lippia javanica* (Endris *et al.*, 2016b). Nonetheless, few studies have been carried out in Kenya to evaluate the aforementioned non-volatile compounds of *L. javanica*.

Even though the phenetics and chemotaxonomic markers contribute a lot to classification, genetic characteristics have been found to be most useful. They have proved to be vital in distinguishing between plant taxa at different levels i.e. cultivars and/or individuals occupying different ecological niches (Sujii *et al.*, 2013). DNA-based analyses have radically influenced nearly all areas of biological research. Consequently, they offer strong evidence in elucidating evolutionary mechanisms, population dynamics, phylogenetic relationships, and systematics (Särkinen *et al.*, 2013). Understanding the genetic diversity within a species is inevitable in the management of genetic resources. This is the basis for taxonomic categorization and conservation. Molecular marker tools such as Inter-Simple Sequence Repeats (ISSR) and Random Amplified Polymorphic DNA (RAPD) are useful in molecular studies. They play a major role in discerning differences between plant species and subspecies (Ng & Tan, 2015). These genetic marker tools have progressively become suitable in genetic diversity studies and in genotypic identification of medicinal plants (Occhiuto *et al.*, 2014). According to Leal *et al.* (2010), an assessment of the *L. javanica* using the ISSR and RAPD markers lacks in characterizing *L. javanica*. Little data and information is available about molecular studies on the aforementioned species by use of DNA markers.

The highly aromatic plant has been researched on for its remedial value. A previous study by Madzimure *et al.* (2011) on *L. Javanica* depicted it to be an acaricidal that control cattle ticks.

This has led to it being used as a cheap supplement in controlling cattle ticks. Many of its uses also relate to microbial infections like coughs or colds. According to Montanari *et al.* (2011), the aforementioned species complex also has wound healing properties thus used to treat skin infections. Many non-volatile compounds have been identified from *L. javanica*. They include an array of phenolic glycosides, flavanones, flavonoids (Mkenda *et al.*, 2015) and volatile oils that have antimicrobial properties. These bio-active compounds are believed to act with synergic effect to deter microbes and pests. Pests, therefore, find it difficult to develop resistance to them, thus they should be researched on further. Plants with pesticidal properties have been investigated for centuries as alternatives to synthetics.

According to Stevenson *et al.* (2014), *Lippia javanica* has been recorded to significantly suppress a wide range of pests such as rape mites that affect tomatoes in the field and weevils that attack grains i.e. maize during storage in Tanzania. Nevertheless, little progress has been made to develop new effective products (Stevenson *et al.*, 2014). There is a paucity of information regarding the use of *L. javanica* extracts to control aphid pests in Kenya. Stevenson *et al.* (2014) notes that research on pesticidal plants has increased overtime, although it has failed to address carrying out tests under realistic field conditions and knowing the exact concentration that should be adopted in controlling field pests.

1.2 Problem statement.

Infra-specific variations have been observed in *Lippia javanica* populations (Verdcourt, 1992). However, the taxonomic status is conflicting in East Africa and Southern Africa region, where two varieties *L. javanica* var. *javanica* and *L. javanica* var. *whytei* are recognized in the latter locality. Morphological variations are exhibited in various important taxonomic characters; for instance, in the number of inflorescence per axil and length of the peduncles. Morphological differences are also supported by variations in chemical composition in the same populations.

Lippia javanica populations from different places have been reported to vary significantly in chemical composition (Endris *et al.*, 2017a). As previously mentioned, even those within the same locality have shown dramatic variation in the chemical constituents. There is lack of well documented information on *L. javanica* morphometrics with an obvious taxonomic conflict that has been partially addressed. Endris *et al.* (2017a) puts forth that profound differences in the essential oils and other secondary metabolites have also been noted in Zimbabwe, Swaziland, and Tanzania populations and a number of chemotypes described. There is, however, paucity of data in phenetic taxonomic studies regarding *Lippia javanica*. The literature is unpublished and the status of the “two varieties” *L. javanica* var. *javanica* and *L. javanica* var. *whytei* remains informal (Kamanula *et al.*, 2017).

In the past, it was quite difficult to classify plant taxa by use of molecular techniques; however, advancement in the DNA markers have circumvented this problem and markers such as restriction fragment length polymorphism (RFLP) simple sequence repeats (SSRs) and microsatellites have been widely employed (Garcia *et al.*, 2004). The phylogeny of Verbenaceae has been resolved; nevertheless, the family is more problematic when it comes to *Lippia* genera (Lu-Irving, 2013). Even though molecular markers give a clear distinction of species and sub-species, published literature about molecular characterization of the *L. javanica* using simple markers (ISSR and RAPD) in Kenya is not available. There is a clear need for a broad, well-resolved molecular hypothesis for *L. javanica*, which has yet to be addressed in detail in molecular studies. Limited information is available regarding molecular characterization of the *L. javanica* in Kenya using DNA markers. Most research on the chemical composition of *L. javanica* complex focus on volatile essential oils. The non-volatile phytochemical compounds have more often than not been left out. These compounds perhaps play a significant role as pest

repellants. Nevertheless, there is paucity of information regarding Kenyan *L. javanica* phytochemical composition that warrants further investigation to determine the bioactive compounds. Mkenda *et al.* (2015) contends that the metabolites from *L. javanica* are responsible for insecticide activity exhibited on the pests; both in the field and in the stores. The use of *L. javanica* as a botanical pesticide, among other applications, by small holder farmers is widespread in East, Central and Southern African region (Mkenda *et al.*, 2015). More research has been done on the species in connection to the medicinal, acaridal and antimicrobial properties. However, the pesticidal efficacy on cowpea field crop pests has not been fully explored. There is little information available on the use of the crude extracts to manage field aphid pests that infest legumes such as cowpea in Kenya. For instance, there is lack of information on the exact concentration to be adopted and the application of the botanical extracts under real field environments. Thus the aim of this present study is to undertake taxonomic study of *L. javanica* using morphological, phytochemical, and molecular approaches and to evaluate its efficacy against attack by aphids on *V. unguiculata*.

1.3 Justification of the study.

This study will attempt to investigate on the taxonomic conflict that exists within *L. javanica* to correctly identify and classify it. The previously mentioned species complex has morphological variations especially in the inflorescence which are taxonomically important characters. As a result, this study will help in eradicating the taxonomic conflict that has been surrounding *L. javanica*. Furthermore, molecular characterization of the lemon bush genotypes will augment morphometric study. The phytochemical compounds of the species will be determined to help in the chemotaxonomic studies and also in the evaluation of its efficacy against the cowpea aphids. The species is also of importance as it has been utilized traditionally and in conventional medicine whereas exploited as an herbal tea. Pests have become a nuisance to farm produce and

have significantly affected production in terms of quantity and quality. As a matter of fact, Mfuti *et al.* (2017) puts forth that farmers from tropical Africa, Kenya included have suffered a loss of more than 50% of their produce due to pest infestation. There is an urgent need by small holder farmers to adopt cultural farming systems. An urgent need for the use of botanical pesticide is inevitable; consequently the high costs of synthetic pesticides make them unavailable to poor farmers who are the majority. They also negatively impact on the environment due to their bio-accumulation. As reported by Mkenda *et al.* (2015), *L. javanica* is among the several plants being used as alternatives to the synthetic chemicals. The plant has proved to be effective in protecting bean in the field (Mkenda *et al.*, 2015). According to Stevenson *et al.* (2014), post-harvest trials have also been shown to be fruitful however, it has unpredictable efficacy. The right concentration to be applied is also unknown. Also, studies fail to test the efficacy of *L. javanica* in real field environment. This current study, therefore, seeks to elucidate the above raised uncertainties about this species complex.

1.4 Objectives

1.4.1 General objective

The purpose of this study was to undertake taxonomic studies on the proposed variants var. *javanica* and var. *whytei*. of *L. javanica* complex based on morphometrics, molecular and phytochemical characteristics, and to evaluate its efficacy as a botanical pesticide for the control of aphids (*Aphis craccivora*) (C.L) Koch of cowpeas (*Vigna unguiculata*).

1.4.2 Specific objectives

1. To determine the morphological variation of *L. javanica*.
2. To determine the molecular characteristics of *L. javanica*.
3. To determine the phytochemical compounds of *L. javanica* leaves.

4. To evaluate the efficacy of different concentrations of *L. javanica* extracts in the control of aphids of *V. unguiculata*.

1.5 Hypotheses

H_a: The morphological features of *L. javanica* exhibit variation.

H_a: The molecular characteristics of *L. javanica* exhibit variation.

H_a: There are diverse phytochemical compounds in *L. javanica* leaves.

H_a: There are significant differences in the efficacy of *L. javanica* in the control of aphids of *V. unguiculata*.

CHAPTER 2: LITERATURE REVIEW

2.1 History of the taxonomy of *Lippia javanica*.

Lippia javanica (Burm F.) Spreng belongs to the Verbenaceae family (Verbenaceae). The family comprises of approximately 32 genera and 840 species. It is a multi-stemmed woody shrub whose height ranges between 0.6 to 4.5 m; it is worth to note that in some circumstances the aromatic shrub grows to the extreme reaching 6 m tall (Verdcourt, 1992). The genus *Lippia* L. is named after Augustin Lippi (1678–1701), an Italian botanist and natural historian who was killed in Ethiopia at the age of 23 (Maroyi, 2017).

Phylogenetic relationships within family Verbenaceae demonstrated that genus *Lippia* and other closely related genera, namely, *Aloysia* Pal'au, *Lantana* L., and *Phyla* Lour are not monophyletic (Maroyi, 2017). The genus *Lippia* is composed of approximately 200 species, shrubs or herbaceous plants, which are widely distributed throughout the tropical, subtropical, and temperate regions of America, Africa, and Asia (Ramos *et al.*, 2011). *Lippia javanica* is one of the African species of the genus *Lippia* popularly known as the “Lemon bush” occurring in central, eastern, and southern Africa. It is an erect, small, woody annual shrub that grows to approximately 4m high. Its habitat is commonly the grasslands, the hillsides, and stream banks. It is also a constituent of the scrub on the fringes of forests (Mkenda & Ndakidemi, 2015). *Lippia* and *Lantana* genera are the most difficult to separate. They show similarities in their inflorescences. Common features being spicate heads, often subcapitate during anthesis and elongating in fruit and pedunculated (Maroyi, 2017).

Lippia and *Phyla* genus have also been treated as one according to a number of botanists. Both the genera have dense spikes that are congested during anthesis. They are often subcapitate, with closely imbricate flowers. However, the Genus *Phyla* differ by often being herbaceous (Gross *et al.*, 2017). It's also characterized by possessing trailing or ascending stems

with roots at the node. Furthermore, spikes are longer than broad bearing cuneate-obovate or flabelliform bracts that are not 4-ranked (Munir, 1993). *Lippia javanica* seems also morphologically similar to *L. scaberrima* Sond but is much taller. The bracts of the later are shorter than the flowers. *Lippia scaberrima* is multi-stemmed and usually grows to less than 0.5 meters in height unlike *L. javanica*.

Lippia alba (Mill) N.E.Br ex Britton and P. Wilson and *L. javanica* were also perceived to be conspecific (Verdcourt, 1993). This led to them being recognized as one species by various botanists. However after studying the type specimen of *L. javanica*, preserved in the Delessert Herbarium at Geneva, they were discovered to be distinct. In the southern African region Kamanula *et al.* (2017) posits that there exist two morphologically distinct varieties, *L. javanica* var. *javanica* and *L. javanica* var. *whytei*. As a result, there is a paucity of data on morphological and molecular characteristics of *L. javanica* species complex.

2.2 Molecular characterization of *Lippia javanica*.

Historically, Verbenaceae classification has proved to be difficult due to the complexity in the form (Lu-Irving, 2013). Broad molecular phylogenetic analyses have proved to be futile in trying to resolve relationships within it. According to Gross *et al.* (2017), recent re-circumscription has shown tribe Lantaneae to be a monophyletic group; the two major genera being *Lantana* L. and *Lippia* L. that encompass seven smaller genera. The two principal genera of Lantaneae constitute the greatest proportion of the species with *Lippia* genera having 200 species and *Lantana* having 150 species (Lu-Irving, 2013).

Most systematic studies today contain phylogenetic investigations based on molecular characters alone or in combination with morphological data (Sørensen *et al.*, 2015). Even with the molecular characterization using markers, it is always recommended to use more than one type of marker for easier comparison (Leal *et al.*, 2010). Taxonomy in *L. javanica* is difficult to

resolve based on morphometrics alone. This is because of the intricate overlapping patterns of shifts in morphological traits among members (Manica-Cattani *et al.*, 2009). Monophyly of the traditional genera cannot be presumed without confirmation with molecular data results. Lu-
Irving (2013) contends that data from multiple and independent loci reveal individual gene trees that are incongruent. Furthermore, Manica-Cattani *et al.*, (2009) contends that neotropical lineages often times referred to as ‘problematic’ taxa are common; problematic taxa comprises plant groups in which traditional classifications are at odds with newly obtained molecular evidence. According to Tamura *et al.* (2013) comparative sequence analyses, performed under the principles of molecular evolutionary genetics, are essential for using this data to build the tree of life. This infers evolutionary patterns of genome and species evolution. They also can elucidate mechanisms of evolution of various morphological and physiological characters (Lu-
Irving, 2013).

With the increasing range of modern tools available to systematics, great progress has been made in the last several years. The evolutionary histories of difficult taxa in important Neotropical families has been untangled (Särkinen *et al.*, 2013). The lineages studied in *Lantaneae* have in common particular characteristics that make it problematic to separate: it is species-rich and geographically widespread (Garcia *et al.*, 2004). Molecular markers such as Random amplified polymorphic DiNucleic Acid (RAPD) markers; Amplified Fragment Length Polymorphism, (AFLP), Inter-Simple Sequence Repeats (ISSR), and Microsatellite have proven to be powerful tools in the assessment of genetic diversity. This has been demonstrated in a number of medicinal and aromatic plants including *Ocimum sp.* L. (Garcia *et al.*, 2004). According to Garcia *et al.* (2004), Random Amplified Polymorphic DiNucleic Acid (RAPD) and Inter simple sequence repeat (ISSR) markers have been used with success. They have been used

in identification and determination of relationships at the species, population and cultivar levels in many plant species like in tropical maize inbred lines (Gross *et al.*, 2017). Consequently the taxa analyzed comprised majorly of aromatic and medicinal plants. A study by Manica-Cattani *et al.* (2009) on the analysis of genetic variation among Brazilian *L. alba* accessions using ISSR and RAPD markers found out that there was a high genetic variability. A total of 120 bands most of which were polymorphic were obtained (84.1%), with the average number of bands per primer being 12. In another study by Ansari *et al.* (2012) using ISSR markers (UBC-801, 834, 880, 899 and 900), 29 populations of teak (*Tectona grandis* L.f) from central and Peninsular India showed 100% polymorphism. UBC-900 recorded the highest Nei's genetic diversity (0.32 to 0.40) and UBC-899 had the highest Shannon's Information Index (0.49 to 0.59). According to Lu-Irving (2013), nine species of *Lippia* excluding *L. javanica* were studied by Random Amplified Polymorphic DiNucleic Acid (RAPD) markers. This was in the evaluation of the genetic diversity among them.

The molecular data were used to generate UPGMA dendrogram that showed two major groups with a clear distribution among the *Lippia* genera accessions (Lu-Irving, 2013). Even though the aforementioned information is available regarding *Lippia javanica*, in Kenya not much has been reported on the use of markers to characterize *Lippia javanica*.

2.3 Chemical composition and variation in *Lippia javanica* plant species.

A wide range of secondary metabolites have been isolated because of the uses they have (Pochapski *et al.*, 2011). The metabolites have been utilized in a number of applications such as in pharmacology, as pesticides, and in aromatherapy among other uses. Viljoen (2007) surmises that these metabolites have been isolated from *L. Javanica* and they seem to differ from region, time, and seasons; surprisingly, they also vary from plants in the same locality (Viljoen,

2007). Major components in the essential oil have been reported to be myrcenone, myrcene, and (E)- and (Z)-tagetone (Mkenda *et al.*, 2014). Accordingly, other components of *L. javanica* are caryophyllene, linalool, and p-cymene (Mkenda *et al.*, 2014).

Kamatou & Viljoen, (2010) reported variations in major essential compounds for *L. javanica* samples taken from the same location in Zimbabwe e.g. linalool, which had a range between 1.7 and 27%. Populations from Swaziland were found to be represented by four distinct chemotypes (Madzimore *et al.*, 2010) in which the chemotypes composed majorly of myrcenone (36.3%), myrcene, and phellandrene compounds. Carvone compound was not detected in the populations, but has been found to occur in other regions in *L. javanica* complex whereas Limonene had (43.4%) and piperitenone with (39.9%) were also reported as major compounds in other populations in the region (Kamatou & Viljoen, 2010). De Marino *et al.* (2012) reported a geranial and neral chemotype from for the first time in Tanzania.

According to Kamatou & Viljoen, (2010), polar extracts also show variation in chemical compounds, with clear differences observed between Swaziland and South African populations (Mkenda *et al.*, 2014). Phenylethanoid, verbascoid, β -[(3, 4-dehydroxyphenyl)-ethyl]-(3'-O- α -L-rhamnopyranosyl)-(4'-O-caffeoyl)- β -D-glucopyranoside, and Isoverbascoside previously known in *L. javanica* have been isolated (Kamatou & Viljoen, 2010). The discovery of these compounds gave a new important insight on the importance of *L. javanica* as a useful plant. *Lippia javanica* have been known to possess antioxidant activity which is still to be investigated. Few chemotypes have been identified in this medicinal plant which includes; myrcenone, carvone, piperitenone, ipsenone, and linalool and the major one being myrcenone (Viljoen *et al.*, 2005).

Multiple classes of non-volatile secondary metabolites, such as alkaloids, amino acids,

flavonoids, iridoids, triterpenes as well as several minerals have been identified from *L. javanica* (Endris *et al.*, 2016a). Total phenolic, tannin content, and radical scavenging activities of *L. javanica* have also been determined (Maroyi, 2017). Semenya & Maroyi (2013) reported tannin content of *L. javanica* as low when compared to *Aspalathus linearis* Rooibos. *Lippia javanica* has higher radical scavenging activity than *A. linearis* which is probably due to higher total phenolic content (Semenya & Maroyi, 2013). The volatile oils and other non-volatiles of *L. javanica* have demonstrated dramatic variations both in quality and quantity within and between natural plant populations. Much literature available regarding *Lippia javanica* chemotaxonomy is about the essential oils and even chemotypes such as myrcenone, carvone, piperitenone, ipsenone have been described (Viljoen *et al.*, 2005) as previously mentioned. However, there is limited research on non-volatile phytochemical composition on Kenyan *L. javanica* populations.

2.4 Pesticidal effects of *Lippia javanica*.

Botanicals have great potential impact in developing countries. A scientific understanding of their activity provides opportunities to optimize their use. *Lippia javanica* is reported to be effective in controlling aphids and red spider mites in cabbage, rapeseed and tomatoes (Muzemu *et al.*, 2011). Its extracts have potential as environmentally friendly alternatives for the control of various insect pests. According to Kamanula *et al.* (2017), few chemotypes have been identified in this medicinal plant which includes; myrcenone, carvone, piperitenone, ipsenone and linalool and the major one being myrcenone. These compounds can be repellent or toxic to highly specific pests or a wide range of organisms (Duke *et al.*, 2010). The use of pesticidal plants against pests has been reported in tropical regions such as southern Africa (Stevenson *et al.*, 2014). This is largely based on ethno-ecological knowledge from the indigenous inhabitants. Stevenson *et al.* (2014) posit that there are efforts to address optimization, sustainability and safety issues in botanical pesticide use. *Lippia javanica* have been reported to be acaricidal by

Zimbabwean smallholder farmers (Madzimure *et al.*, 2011). Studies have evaluated the repellent effects of hexane extracts of *L. javanica* essential oil using the *in vitro* tick climbing repellency bioassay on adults of *Hyalomma marginatum rufipes* Koch ticks. Madzimure *et al.* (2011) found out that 107 mg/ml caused repellency index of 100% at one hour and 30 minutes. In a similar study by Madzimure *et al.* (2011), *L. javanica* aqueous leaf extract at 10% and 20% w/v were effective at controlling cattle ticks (*Amblyomma* species C.L, *Boophilus* species Lahille, *Hyalomma* species L., *Rhipicephalus appendiculatus* Koch, and *Rhipicephalus evertsi* Koch). They were as found out to be good as the positive control amitraz-based acaricide Trickbuster® (Madzimure *et al.*, 2010).

The volatile oils of *L. javanica* were reported to have antimicrobial properties (Endris *et al.*, 2016b). Despite the widespread use of *L. javanica* for medicinal and acaricidal purposes (Sola *et al.*, 2014), not much has been unearthed in literature about its potential as a botanical pesticide more so the exact concentration and correct use. Furthermore, *L. javanica* is found locally, it is non-toxic and its extracts do not persist to the environment. However, in Kenya few studies have focused on applying the botanical extracts in control of cowpea aphid pests (Muzemu *et al.*, 2011). This calls for further studies on how this plant can be utilized effectively in controlling common field pests that attack *Vigna unguiculata* in the field.

CHAPTER 3: MATERIALS AND METHODS

3.1 Study site

Morphological study of *Lippia javanica* was conducted in the East African (EA) Herbarium at the national museums of Kenya (NMK). Genetic variation of the *L. javanica* accessions was done in the molecular laboratory of Masinde Muliro University of Science and Technology, Kenya (MMUST). Phytochemical compounds were determined at the Phytochemistry Laboratory of Maseno University, Kenya. The study site for pesticidal efficacy was conducted in Makueni County (Nzouni, village). The study was conducted from November 2016 to February 2017 growing season on Mrs. Patricia Mukai farm. The region where the study was conducted lies between S 02⁰⁰.877 E 37.862569 (Figure 3.1). The elevation is 920 M above sea level (asl). There are two rain seasons; the long rains occurring in March to August while the short rains occur in November to December while the rest of the year is hot and dry. The general mean annual rainfall is 591 mm with average temperature of 24.1°C.

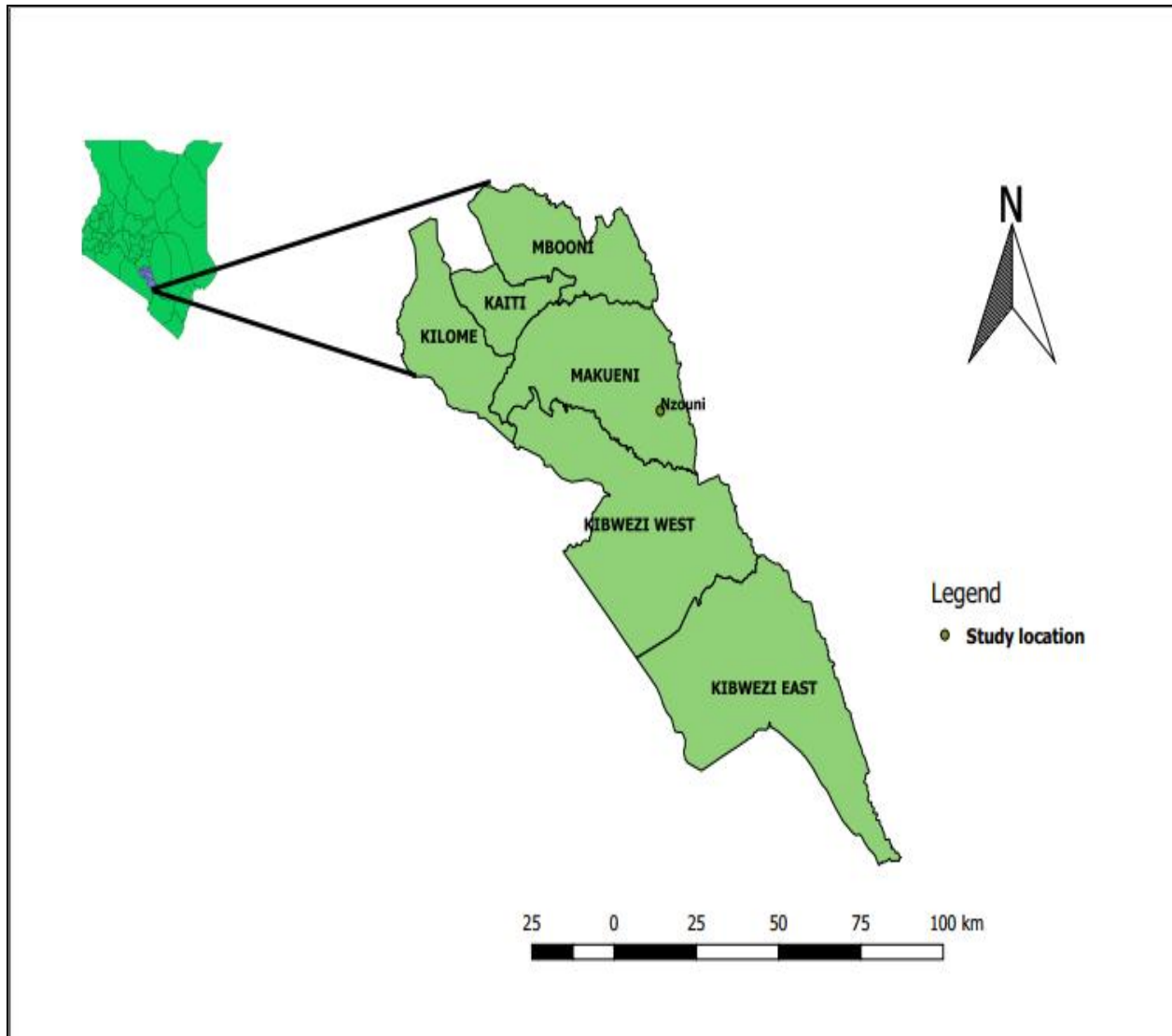


Figure 3.1: Study area showing Nzouni village, Makueni County where field experiment on efficacy trials of *L. javanica* extracts was conducted.

(Source: Google map July, 12th, 2016).

3.2 Morphological characterization of *L. javanica*.

Specimens of *L. javanica* used in studying morphological features were accessed from the EA Herbarium, NMK, Nairobi. The voucher specimens were from different collectors with their respective numbers, coordinates and other relevant data (Appendix 1). Kenyan, Tanzanian, and Ugandan specimens were examined and measurements taken from them by a millimeter hand ruler and a calibrated objective lens of the WILD M3 microscope. The type specimen was loaned

from the Royal Botanic Gardens, Kew (K), K000379290 and was used in the confirmation if they matched the species description. Specimens studied in the EA herbarium were according to the extract from the Botanical Research and Herbarium Management Systems (BRAHMS) data base. Specimens collected from the field were incorporated to augment the studies. The field specimens were collected from Maralal (N 01°05.080 E 036.41.225), Naivasha (S 01°00.822 E 036°19.994), Nyahururu along Rumuruti (N 00°07.889 E 036.23.581), Sotik (S 00°23.510 E 035.01.957), Sondu (S 00°23.512 E 035.02.065), Kedong (S 01°00.853 E 036.20.013), Kapsowar (N 00°58. 670 E 035.33.978), Ngong in Kona Baridi (S 01°46.252 E 036.65.121) and Narok (S 01°08.504 E 035.5.654). A snap shot of the *Lippia javanica* while in the field at Ngong, Kona baridi, Kajiado County is as shown in plate 3.1. In each locality visited, five voucher specimens were randomly obtained where the distance from one to the other was a minimum of 100 meters away to avoid collecting related *L. javanica* populations. Label information was recorded i.e. flora area/locality, species details (name, family, habit/description, frequency, and uses), habitat, collector (s) and number (voucher number). Voucher specimens from the field were pressed according to the standard procedures (using plant press and drier) and transported while in the plant press (Bridson & Forman, 2010). Upon arrival, they were placed in the drier for three days and were removed for mounting of the prepared specimen labels. They were then laid in the EA herbarium cabinets in their rightful place. It should be notable that *Lippia javanica* was identified if it matched the descriptions according to Verdcourt (1992). A sample of the prepared herbarium voucher specimen is as shown in appendix 2.

3.2.1 Data collection on the *L. javanica* specimens.

Voucher specimens studied amounted to 63 although only 52 were selected in the final analysis. This was due to immaturity and incompleteness of some characters, i.e. lack of fully open florets and not fully developed inflorescence. Out of the thirty six quantitative characters, only twenty

qualified for inclusion in the analysis as they were complete unlike the sixteen which had some discontinuities (Otieno *et al.*, 2006b). Eight qualitative characters were used in the description of *L. javanica* characteristics.

The consideration of the specimens included the locality, those with fully mature parts such as flowers, leaves and stems. This was done so as to avoid biases that may arise from developmental plasticity. This also allowed for standardized measurements to be taken for all the specimens thus avoiding bias. The material selected also qualified for inclusion if it matched the descriptions and key characters of *L. javanica* according to (Munir, 1993; Verdcourt 1992). The field studies were confined to regions of Kenya where the greatest variability of *L. javanica* are known to occur by checking the measurements that had been obtained from the herbarium specimens. The specimens were studied using a WILD M3 dissecting microscope under $\times 10$ and $\times 40$ magnifications. Prior to examining, the floral parts of the preserved specimens were rehydrated in warm water to soften them before dissecting them. However, those direct from the field were not rehydrated as they were fresh. Out of the 36 meristic characters scrutinized, only 20 were found appropriate in the subsequent phenetic analyses. Eight non-meristic characters corded as binary characters were also examined. Ten measurements of each quantitative character were taken per specimen using a hand ruler calibrated in mm. Small parts were measured using a calibrated eye piece lens of a WILD M3 dissecting microscope under at $\times 10$ mg and $\times 40$ mg objectives.



Plate 3.1: *Lippia javanica* in the field at Kona Baridi, Kajiado County. (Photo by: Collins, May, 26th, 2016).

3.3 Molecular characterization of *L. javanica* accessions.

Lippia javanica leaf materials freshly collected from the field were used. The leaf materials were from selected different five localities (Table 3.1) in Kenya to test if any molecular variants occurred. Three pieces of the leaf materials were stored in zip lock bags containing silica gel and were packed in plastic bags and kept in a container where they were ferried to the laboratory for storage in a cool dry place in the laboratory. DNA was extracted from the leaves, cleaned and thereafter subjected to ISSR and RAPD PCR (Manica-Cattani *et al.*, 2009).

3.3.1 Collection and preservation of tissue materials from *L. javanica*.

Three fresh pieces of young leaves (green healthy leaves) were collected from selected plants in the floral areas of Kenya as shown in Table 3.1. The plant materials were kept in plastic bags and track of individual plant from which sample materials were sampled and kept by labeling with small tags. The leaf materials were cut into smaller pieces. The leaf tissues were kept into small sealable bags containing silica gel. The ratio of silica gel to plant tissue was 10:1 (Manica-Cattani *et al.*, 2009).

The collection numbers of the individual plant were written on a small piece of paper and inserted in the Ziploc bag. The information was also written on the outside of the bag with a permanent marker. The silica gel packets were stored in a sealed bag to keep moisture out. Care was taken to ensure that they did not rehydrate over time. The plant from which the sample was taken from was pressed and processed using the standardized herbarium procedures and deposited in the E. A Herbarium, NMK. The field specimens were collected to supplement the information obtained from the stored herbarium specimens.

Table 3. 1: *Lippia javanica* accessions that were collected and selected for analysis for genetic variability and their corresponding floral region.

Origin	Voucher Name & No.	Coordinates
1. Maralal	Masinde C.W & M.F 0037A	N 01°05.080 E 036.41.225
2. Maralal	Masinde C.W & M.F 0037B	N 01°05.080 E 036.41.225
3. Kedong	Masinde C.W & M.F 0025	S 01°00.853 E 036.20.013
4. Kedong	Masinde C.W & M.F 0029	S 01°04.237 E 036.97.011
5. Nyahururu	Masinde C.W & M.F 0035	N 00°07.889 E 036.23.581
6. Nyahururu	Masinde C.W & M.F 0036	N 00°03.845 E 036.57.621
7. Sondu	Masinde C.W & M.F 0019	S 00°23.512 E 035.02.065
8. Sondu	Masinde C.W & M.F 0020	S 00°17.413 E 035.19.032
9. Narok	Masinde C.W & M.F 0038	S 01°08.504 E 035.5.654
10. Narok	Masinde C.W & M.F 0039	S 01°03.128 E 035.7.123

3.3.2 DNA extraction.

Plant tissue (leaf) of each selected plant was ground separately under liquid nitrogen (lysis) to a fine powder using a mortar and pestle (Manica-Cattani *et al.*, 2009). The tissue and liquid nitrogen were transferred to an appropriately sized tube and liquid nitrogen allowed to evaporate. The sample was not allowed to thaw and was preceded immediately to the DNA preparation protocol PCR (Manica-Cattani *et al.*, 2009).

5 ml of Buffer AP1 (preheated to 65°C) and 10 µl of RNase A stock solution (100 mg/ml) was added to a maximum of 1 g of ground tissue and vortexed vigorously. It was ensured that no tissue clumps was visible. Vortexing or pipetting was further done to remove any clumps. The mixture was incubated for 10 minutes at 65°C and mixed 2–3 times during incubation by inverting the tube. 1.8 ml of Buffer AP2 was added to the lysate, mixed, and incubated for 10

minutes on ice. Lysate was centrifuged at 3000–5000 x *g* for 5 min at room temperature. A pellet formed, but some particles floated. The supernatant was decanted into the QIA shredder Maxi Spin Column (lilac) placed in a 50 ml collection tube and was spinned at 3000–5000 x *g* for 5 minutes at room temperature (15–25°C) in a swing-out rotor (Manica-Cattani *et al.*, 2009).

The flow-through was transferred without disturbing the pellet in the collection tube, to a new 50 ml tube, and the volume recorded. Typically, 6 ml of lysate was recovered. After centrifugation of the sample, most of the debris and precipitates was retained in the filter but there was also a pellet in the collection tube. Care was taken to avoid disturbing the pellet when transferring the supernatant. 1.5 volumes of Buffer AP3/E were added directly to the cleared lysate and mixed immediately by vortexing. The sample was applied to the DNeasy Maxi Spin Column (colorless spin column) including the precipitate which formed (maximum loading volume 15 ml). It was centrifuged at 3000–5000 x *g* for 5 minutes. 12 ml Buffer AW was added to the DNeasy Maxi Spin Column and centrifuged for 10 min at 3000–5000 x *g* to dry the membrane. The flow-through and collection tube were discarded. The DNeasy Maxi Spin Column was transferred to a new 50 ml tube and 1 ml of Buffer TAE pipetted directly onto the DNeasy Maxi Spin Column membrane and left for 5 min at room temperature (15–25°C). It was centrifuged for 5 min at 3000–5000 x *g* to elute. Another 1 ml of Buffer AE was added and the elution repeated as described in the above step (Guilliam *et al.*, 2017).

3.3.3 Amplification of DNA by Polymerase Chain Reaction (PCR).

A 1 % solution of agarose was prepared by melting 1 g of agarose in 100 mL of 0.5x TAE buffer in a microwave for approximately 2 minutes. It was allowed to cool for a couple of minutes and then 2.5 µl of ethidium bromide was added, and stirred to mix. A gel was casted using a supplied tray and comb. The gel was allowed to set for a minimum of 20 minutes at room temperature on a flat surface. 10 µL 1kb ladder and 5 µL sample + 5 µL water + 2 µL 6x Loading

Buffer was loaded into separate wells. The gel was run for 30 minutes at 100 V and exposed to UV light and photograph taken (Sample of the UV-trans-illuminator during gel observation is in appendix 3). The DNA quality was confirmed by the presence of a highly resolved high molecular weight band (Manica-Cattani *et al.*, 2009).

3.3.4 Evaluation of genetic variability using ISSR and RAPD markers.

The RAPD amplification reactions (final volume of 25 μ L) contained: 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 200 μ M of each dNTP, 1.5 mM MgCl₂, 50 μ M primer, 1 U Taq-polymerase, and 25 mg of plant DNA. ISSR reactions were similar except for primer concentration (30 μ M) and 0.8 U Taq-polymerase.

The amplification conditions for ISSR and RAPD were at an initial step of 5 minutes at 94 °C followed by 40 cycles of 1 minute at 94 °C for denaturing, 45 s at 40 °C (RAPD) or 48 to 50 °C ISSR for annealing, and 2 minutes at 72 °C for extension, and a final extension of 5 minutes at 72 °C.

Amplification products were resolved by electrophoresis in 1.5% (w.v-1) agarose gels in TBE buffer under a constant voltage of 90 V. DNA fragments were stained with 1 μ g.mL⁻¹ ethidium bromide and digitalized under UV light for further analysis. Bacteriophage Lambda cut with *Eco*RI and *Hind* III were included as size standard on each gel.

The bands were assigned molecular weights based upon their positions relative to a Lambda molecular weight standard. ISSR and RAPD amplicons were scored for presence or absence in each accession, and the data were entered into a binary matrix as discrete variables ('1' presence and '0' absence) as in (Appendix 4), (Manica-Cattani *et al.*, 2009).

3.4 Determination of the qualitative phytochemical compounds of *L. javanica*.

Lippia javanica leaf material from different samples (Table 3.1) were cleaned and dried separately in the open air under shade for a one week period. They were then crushed into fine powder using Kika Werke M20 grinder.

The crude extracts were prepared by modified method of Stevenson *et al.* (2014). Three hundred grams of the powder was weighed using a weighing balance and soaked in 1200 mL of ethyl alcohol (80% BDH), at a ratio of 1:4 (powder/ solvent). The mixture was then agitated using an electric blender (to enhance proper mixing of the solvent with powder) and then poured in an air-tight plastic container. The containers with the mixture were then kept in the refrigerator at 4°C for 48 hours. The mixtures were then filtered using cheese cloth, then with Whatmann No. 1 filter paper. Then they were separately concentrated in vacuo using Rotary Evaporator (Model RE52A, China) to 10% of their original volumes at 37 °C-40°C. These were concentrated to complete dryness in a water bath. The extracts were tested for the presence of bioactive compounds by following the standard methods adopted from Nalubega *et al.* (2014).

3.4.1 Test for flavonoids and flavones

Five ml of the crude extract of *L. javanica* was mixed with few fragments of magnesium ribbon. 2ml of concentrated HCl was added drop wise. Formation of a pink scarlet color was indicative of the presence of flavonoids and flavones.

3.4.2 Alkaline reagent test

Two ml of the crude extract of *L. javanica* was mixed with 2ml of 2% solution of NaOH and then a few drops of dilute sulphuric acid were added; an intense yellow color which turned colorless showed a positive result.

3.4.3 Test for terpenoids

Five ml of the crude extract of *L. javanica* was dissolved in 2ml of chloroform and evaporated to dryness. Two ml of concentrated H₂SO₄ was added and heated for about 2 minutes. A gray color indicated presence of the terpenoids.

3.4.4 Test for phenols

Two ml of distilled water was added to 1mg of plant sample, followed by 10% of aqueous ferric chloride solution. A blue coloration showed a positive test.

3.4.5 Test for phenolic glycosides

Two mg of the plant extract sample was dissolved in 1ml of water then aqueous NaOH added. Formation of yellow color indicated the presence of the phenolic glycoside while persistence in the color of the crude extract showed the absence of the phenolic glycoside.

3.4.6 Test for cardiac glycosides

One hundred ml of extract of *L. javanica* was dissolved in 1ml of glacial acetic acid containing one drop of ferric chloride solution. This was then under-layered with 1 ml of concentrated H₂SO₄. Appeared of a brown ring at the interface of the two layers where the lower acidic layer turned blue green upon standing was indicative of presence of the cardiac glycosides (Appendix 5).

3.4.7 Test for saponins

Crude extract was mixed with 5 ml of distilled water in a test tube. The test tube was shaken vigorously. Formation of stable foam showed a positive result.

3.4.8 Test for alkaloids

Five ml of the crude extracts was mixed with 2 ml of 1% HCl and heated gently. Mayer's and Wagner's reagent were then added to the mixture. Turbidity of the resulting precipitate inferred the presence of alkaloids.

3.4.9 Test for resins

Five ml of acetic anhydride was added to 2 mg of plant extract in a test tube, the resulting mixture was dissolved by gentle heating. After cooling, 0.5ml of H₂SO₄ was added. Formation of a bright purple color was indicative of resins presence while absence of the purple color showed the absence of resins.

3.4.10 Test for anthraquinones

One gram of the extract was dissolved in 70% acetone to a final concentration of 50mg/ml. The Bonträger test will was to test for anthraquinones. 2 ml of the test sample was shaken with 4 ml of hexane to defat. The upper lipophilic layer was separated and treated with 4ml of dilute ammonia. The formation of two layers; in which the lower one turned to violet and thereafter turned pink was indicative of the presence of anthraquinones.

3.4.11 Test for Coumarins

Coumarins were tested by dissolving the residue by heating in 2 ml of water. 0.5 ml of 10% ammonia solution was added. The presence of a blue fluorescence under ultraviolet light showed a positive reaction for the presence of coumarins.

3.4.12 Test for Tannins

Tannins were detected using Braemer's test 15. To differentiate between the tannins, three drops of ferric chloride were added to 1 ml of methanol and of the aqueous extract. A black to red colour change was a confirmative test for the presence of tannins.

3.4.13 Test for Polynuroides

About 2 ml of aqueous extract was added drop wise to 10 ml of ethanol. There was formation of a thick precipitate. The formed precipitate was further separated off and washed away with ethanol and stained with methylene blue. Formation of violet color was indicative of polynuroids presence whereas absence of the violet color indicated the absence of polynuroids.

3.5 Efficacy of the extracts of *L. javanica* in control of aphids of *V. unguiculata*.

3.5.1 Plant materials collection and processing.

Fresh leaves of *L. javanica* were collected from different localities around Kenya where the species occurs because the already stored herbarium pressed specimens could not be used for molecular studies. Voucher specimens and Geographical Positioning Systems (GPS) coordinates were lodged at the East Africa Herbarium Nairobi National Museum. The species (*L. javanica*) was chosen for this study due to its wide abundance around farms, roadsides and bush land. It is also familiar to farmers and considerable knowledge on its efficacy exists. Leaves were dried under shade for a week and then ground into fine powder using a grinder. The powders were labeled and then stored in black plastic bags under dark and dry conditions until they were required (Mkenda *et al.*, 2015).

3.5.2 Field preparation and cowpea planting at Makueni County, Nzouni village.

The field at Makueni was disc harrowed and ridged prior to planting. The common cowpea (*Vigna unguiculata*) seeds used for planting were of the variety Katumani KVU 27-1. They were obtained directly from the breeder at Kenya Agricultural and Livestock Research Organization (KALRO) Katumani, Machakos. The seeds were planted at a spacing of 50 cm between rows and 20 cm within rows in 3 x 4 m plots which were 1 m apart. 3 seeds were seeded per hole and then thinned to two plants one week after germination. The experimental layout was a randomized complete block design, and the treatments were replicated on 4 blocks, all within the same field location (Table 3.2).

Table 3.2: Experimental layout displaying the completely randomized block design.

5% 1001	10% 1002	+Ve 1003	1% 1004	-Ve 1005
1% 2001	-Ve 2002	10% 2003	5% 2004	+Ve 2005
10% 3001	5% 3002	1% 3003	+Ve 3004	-Ve 3005
-Ve 4001	+Ve 4002	5% 4003	10% 4004	1% 4005

3.5.3 Preparation of the three concentrations of the botanical extracts and field treatments.

To determine effective pesticidal concentration three different concentrations of the plant extract were made 10%, 5%, and 1% w/v. For 1% solution, 1 litre of water was added to 10 grams of powder, 5% solution was prepared by adding 50 grams of the plant powder to a litre of water, while 10% solution was added by adding 100 grams of the powder to a litre of water. As extractions were carried out in water, a second variable of adding 0.1% soap (which was prepared by adding 1 litre of water to 1 g of panga bar soap whose chemical composition was water-soluble sodium stearate and fatty acids) during extraction was also included to all the treatments. Soap was added as it is known to increase extraction of non-polar compounds and acts as a surfactant during application (Mkenda *et al.*, 2015). The extracts were kept in the buckets to extract at ambient temperature ($20\pm 5^{\circ}\text{C}$) overnight. Thus there were 5 treatments 1%, 5%, and 10% plus positive and negative controls. Each was replicated four times, thus giving 20 blocks. Extracts were kept in 10 L buckets with lids in the shade and filtered through a fine cloth to remove all plant materials that may inadvertently clog the sprayer. The positive control in the trial was synthetic pesticide Atom (Osho, Syngenta). All treatments were replicated across four blocks. The number of aphids before the treatments were counted and found to have an average

of eight. All treatments were sprayed throughout the growing season at an interval of 7 days starting one week after cowpea plant emergence. A 15-litre knapsack sprayer was used to apply the various treatments. The sprayer was thoroughly cleaned with soap and water prior to being re-filled with another formulation for application.

3.5.4 Sampling of aphid infestation.

All assessments were carried out the day before treatments were to be sprayed following the modified method of (Mkenda *et al.*, 2015). The target insect pests to be evaluated were cowpea aphids (*Aphis craccivora*). Three inner rows from each plot were selected for sampling. Due to often very high numbers, a categorical index was used to assess aphid abundance according to the modified method by Mkenda *et al.* (2015).

0 = None;

1 = A few scattered individuals;

2 = A few isolated colonies;

3 = Several isolated colonies;

4 = Large isolated colonies; and

5 = Large continuous colonies.

The severity or degree of infestation in each infested plant was assessed by scoring the extent of damage using scoring grades below according to Mkenda *et al.* (2015). After spraying with all the extracts, a similar procedure of sampling was conducted again the following day in the morning. The difference was taken to be the number of aphid suppressed where it was recorded.

0 = No damage;

1 = Showing damage up to 25%;

2 = Damage from 26%-50%;

3 = Damage from 51%-75% and

4 = Damage more than 75% (Mkenda *et al.*, 2015).

3.6 Data analysis.

A data set was constructed from the measurements obtained from the accessions that were examined. The matrix comprised of all the 63 specimens representing the *L. javanica* complex. Multivariate data analyses were applied on the data using STATISTICA Release 7. The following analyses were performed: Cluster analysis (CA) and principal component analysis (PCA). Cluster analysis was performed to show dissimilarities in the operational taxonomic units (OTU's) (specimens in this case) and also to establish if the data grouped them to discrete clusters; and also, PCA was carried out to further examine the pattern of relationships in the OTU's as well as characters employed (Otieno *et al.*, 2006a). The OTUs were clustered based on Unweighted Paired-Group Method Averages (UPGMA) and their degree of similarity measured by L2 dissimilarity measure. The data was standardized to remove effects of characters with large variances before performing the analyses (Otieno *et al.*, 2006a). Differences among treatments in number of aphids and severity of damage were assessed by analysis of variance (ANOVA). Means were separated by Least Significant Difference (LSD) test at 5% probability level. Analyses were performed in STATA analytical package.

CHAPTER 4: RESULTS

4.1 Morphological variation of *L. javanica*.

All the *Lippia javanica* specimens were found to possess stems with strigose, strigose-villous, and short stiff tubercle –based hairs and small glands. The glands were either pinkish or clear in color. Leaves were opposite and decussately arranged or in whorls of 3. The blades were lanceolate, elliptic, or ovate-lanceolate. The size ranged from 1-6.5 cm long and 0.3-4.2 cm wide obtuse, rounded to acute at the apex, cuneate, obtuse, cuneate-obtuse or rounded at the base; the margins are serrulate. The leaf surface was densely pubescent with tubercle-based hairs and shorter hair above. The hairs were found to be slightly strigose to short distinctly scabrid. They were also rugulose and bullate, softly and densely often more or less velvety adpressed (lying flat on the surface) pubescent beneath and raised nerves, also with small glands that are either colorless or pinkish.

Flowers were in conical, conical-oblong or oblong spikes 0.45-1.5 cm and to the extreme those found at Kedong and Narok measured to 2.2 cm. long, (3-) 5-8 mm. wide, 1-4 per axil, almost sessile or with peduncles 0.3-3 cm. long. The lower bracts of spikes were ovate with an acuminate apex, 4 mm. long, 2-lobed, densely spreading white pubescent and glandular. The Calyx was found to be more or less 1 mm. long, 2-lobed densely spreading pubescent. Corolla white, yellowish white (cream) (Tables 4.1, 4.2, and 4.3 respectively).

Table 4.1: List of 20 OTUs alongside the 8 quantitative morphological characters of *L. javanica* examined.

OTU	LPLB	LPAB	LLBA	LWC	SIR	LWNA	IPA	PLAB	LIBT
KO/1059	35	2.83	32.2	8.8	3.98	5	3	8.1	8.5
MA	35	2.8	32.2	9.2	3.80	6.2	3	3.97	6.15
/GBK003									
MI/756	39.29	2.43	36.86	12.67	3.10	7.57	2	12.86	6.57
K&	47.5	4.33	43.17	13.67	3.47	10.5	2.33	7.67	5.58
M/6037									
WE/2873	30	6	27	10	3.00	5.83	2	6.08	5.75
G&S/3124	44.5	1.33	43.17	16.67	2.67	10.83	3	8.83	7.5
MY/2033	40.7	3.2	37.7	9.9	4.11	4.5	2.2	33	10.7
PO/247	63.2	3.6	59.9	16	3.95	11.14	2	7.1	7.5
MO/49	57.25	3.08	53.67	18	3.18	8.5	2.33	7.11	6.33
IA/529	57.3	3.9	53.4	14.2	4.04	9.1	2	5.7	10.3
HL/127	27.3	3	24.3	9.5	2.87	6.8	1	28.8	11.3
VT/304	34.1	2.4	31.7	10.9	3.13	6.65	3	10.85	5.15
KY/188	49.7	3	46.17	12	4.14	7	3	19.17	7.75
R&C	22.25	1	21.25	10	2.23	5.83	3	1	5.5
/J/11									
IS/2517	35.83	2.17	33.67	8.52	4.21	5.5	2.17	2.33	8.33
CY/52	44.83	1.5	43.33	14	3.20	7.33	3	16.67	9
WA /1622	58.17	2.33	55.83	16.76	3.47	8.5	4.33	16.67	7.5
MI/726	32.42	2.92	29.67	15.42	2.10	10	2.17	4.42	6.17
	33.8	3.6	30.2	9.6	3.52	6.8	2	3.2	4.3
P&K/9356									
H&T/263	46.67	4.33	42.33	11.5	4.06	8.33	2	10.17	7.42

KEY:

OTU – Operational Taxonomic Unit

SIR -Spherical index ratio

LPLB -Length of petiole plus the leaf blade

LWNA -Leaf width near the apex

LPAB -Length of the petiole from the axil to leaf base

PLAB -Peduncle length from axil to the base of inflorescence

LLBA -Length of leaf blade from base to apex

LIBT- Length of the inflorescence from base to tip

LWC -Width of leaf blade at the center

Table 4.2: List of 3 quantitative morphological characters and part of the *L. javanica* OTUs, in which the WILD M3 microscope was used in their observation and values scored using a calibrated objective lens under $\times 10$ mg.

OTU	LKBT	LCBT	WCBP
KO/1059	0.95	1.7	0.92
MA /GBK003	0.8	2.08	0.9
MI/756	1.12	3.07	0.93
K& M/6037	1	1.88	0.83
WE/2873	1.08	2	0.83
G&S/3124	1.42	2.54	0.88

KEY:

LKBT -Length of the corolla tube from base to the tip

LCBT -Length of the calyx from base to tip

WCBP -Width of the corolla tube at the broadest part

Table 4.3: List of qualitative morphological characters found to be possessed by *L. javanica*.

Character	Character state
I. SIH	Strigose , villous, intermediate
II. SS	Squarish, round, 3-angled
III. LPA	Opposite-decussate, whorled in 3s
IV. LS	Lanceolate, Ovate-lanceolate, Ovate
V. LTBA	Strigose , Scabrid
VI. LNGA	Nerves raised with colorless and pinkish glands
VII. FSS	Concoid, Conical-Oblong or Oblong
VIII. SUBS	Acuminate apex Not-acuminate

KEY:

SIH= Stem indumentums

SS= Stem shape

LPA= Leaf phylotaxy

LTBA= Leaf tubercle based hairs

LNGA= Leaf nerves and glands

FSS=Flower spike shape

LS= Leaf shape

SUBS= Shape of upper bract of spike

4.1.1 Cluster analysis for the morphological variation.

Cluster analysis was performed using the quantitative morphological data. All the *L. javanica* specimens revealed two main clusters A and B at 42.5 L2 dissimilarity measure (Figure 4.1). The two clusters were not distinct, thus it was attributed to altitudinal and environmental factors such as climate and soil types. The leaf characters which are usually variable in size were the ones that brought about these variations; and also, cluster analysis has been known to insert a hierarchical structure on the specimens even if the variation is clinal. In fact the supposed varieties were found to cluster together which further confirmed that they were not distinct specimens. A number of specimens from higher altitudes (> 3000 m asl) tend to cluster together.

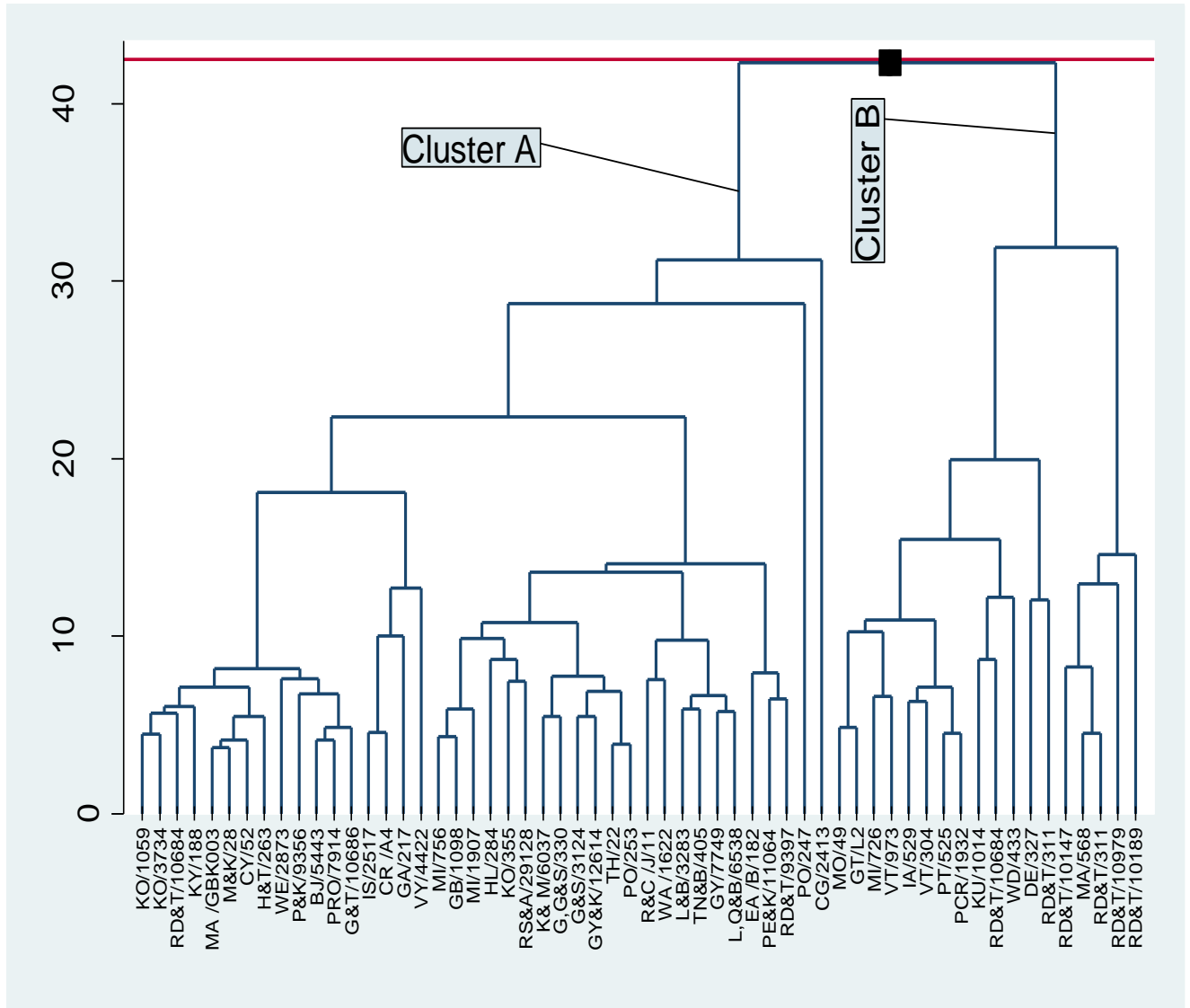


Figure 4. 1: Dendrogram from cluster analysis (UPGMA) of the *L. javanica* specimens. Each OTU has been assigned the first and last letter of the collector and a collector’s number. The specimens are according to the BRAHMS data base of the EA herbarium.

4.1.2 Principal component analysis (PCA) on morphological variation.

To further examine the variation in *L. javanica* complex, Principal Component Analysis (PCA) was performed on the data. PCA is oftentimes used to reduce a large set of variables to a small set but no information is lost in the process (Otieno *et al.*, 2006a). Thus, it was carried out to examine the pattern of relationship between OTUs (specimens in this case). The first component axis was derived to encompass the highest percentage of variation among objects. Similarly, the

second, the third, and remaining component axes were derived to explain the highest percentage of variation left after derivation of previous axes.

The first two factor coordinates in this PCA explained 42.5% of the variation within the data while the third accounted for further 11.2%. The main factors for separation of these specimens were thus alluded to be altitudinal and the aforementioned environmental factors. (Cron *et al.*, 2006). Other major factors contributing to variations were the sizes of the leaf (LPLB, LLBA, LWC, and LWNB), (Table 4.4). Along this factor- plane, the distributions of individuals of the complex were dispersed equally with no signs of morphologically distinct species in the multivariate space. Second factor coordinate similarly exhibited some degree of separation around the multivariate space with no distinct groups as there was an overlap of specimens.

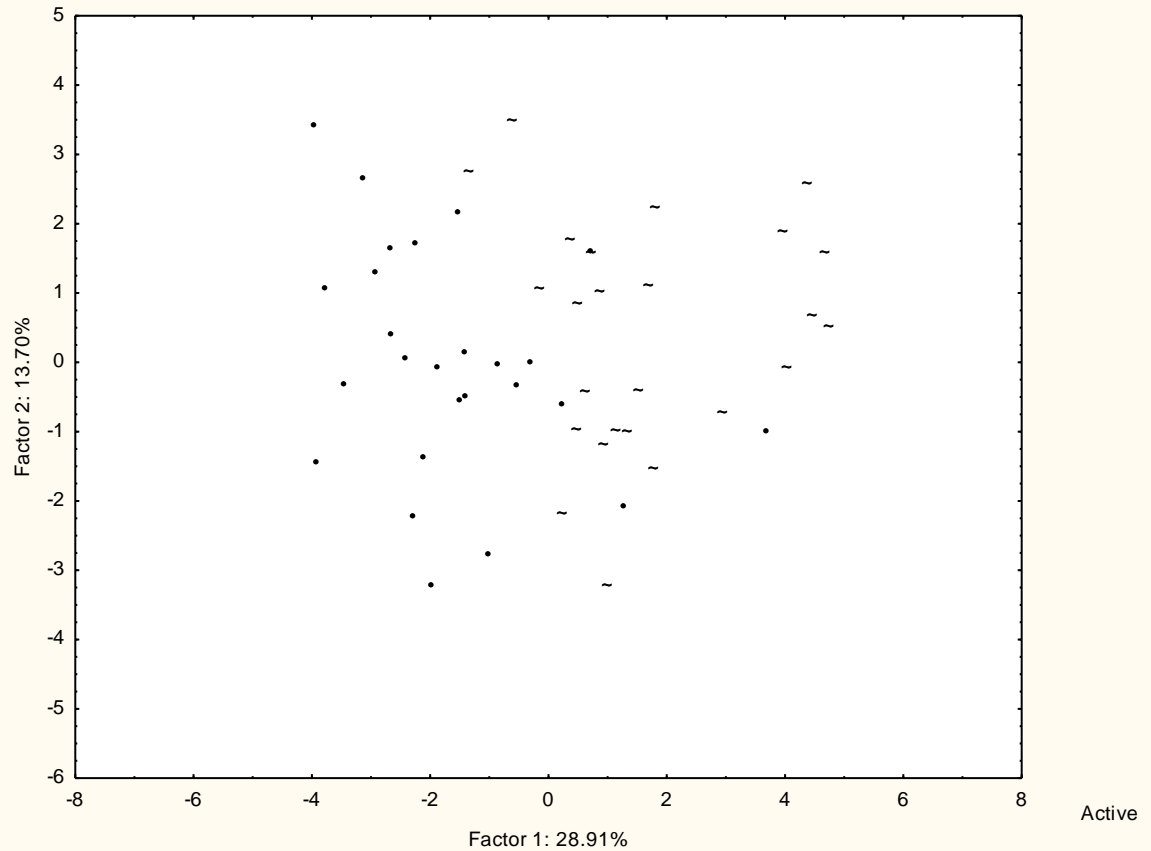


Figure 4.2: Scatter plots of the 52 OTU's of *L. javanica* plotted against the first factor-plane by the second factor-plane. ~ = *Lippia javanica* specimens form1 • = *L. javanica* specimens second form.

Table 4. 4: Factor loading on the first 2 factor coordinates for 20 morphological characters of the *L. javanica*, used in the final PCA. Variables with high loadings on each of the principal components are indicated boldfaced.

Variable	Factor 1	Factor 2
LPLB	0.915647	-0.196835
LPAB	0.490922	-0.267682
LLBA	0.911725	-0.187797
LWC	0.828519	-0.339856
LWNA	0.739811	-0.431997
LWNB	0.851971	-0.368880
SIR	0.396764	0.208612
IPA	0.349220	0.305307
PLAB	0.388736	0.323096
LIBT	0.485897	0.021058
WIB	0.584259	0.084861
WIC	0.435796	0.464837
WINT	0.321302	-0.041996
LLBS	0.573361	0.444675
WLBS	0.153340	0.245858
LUBT	0.379175	0.555232
WUBS	0.057203	0.506867
LKBT	0.268635	0.442281
LCBT	0.36331	0.471679
WCBP	0.022052	0.697173

4.2 Molecular characterization of *L. javanica* accessions.

Genomic DNA obtained from individual accessions Sondu, Nyahururu, Narok, Kedong and Maralal were used as template to perform the analysis using ISSR and RAPD markers. Out of the 6 ISSR primers of ISSR method four primers were selected as they produced robust bands. For RAPD analysis, all of them were selected for further analysis. Primers that were chosen for both the methods produced robust and reproducible bands varying in numbers. For the primers selected, band scores did not differ between repeated experiments or between gels. An example of pattern produced by GACA₄ and OPZ-11 are as shown in the (Plate 4.1 and 4.2) respectively. The ISSR and RAPD primers used to amplify all the accessions yielded 12 and 15 fragments, respectively; resulting in a total of 27 bands which were all monomorphic.

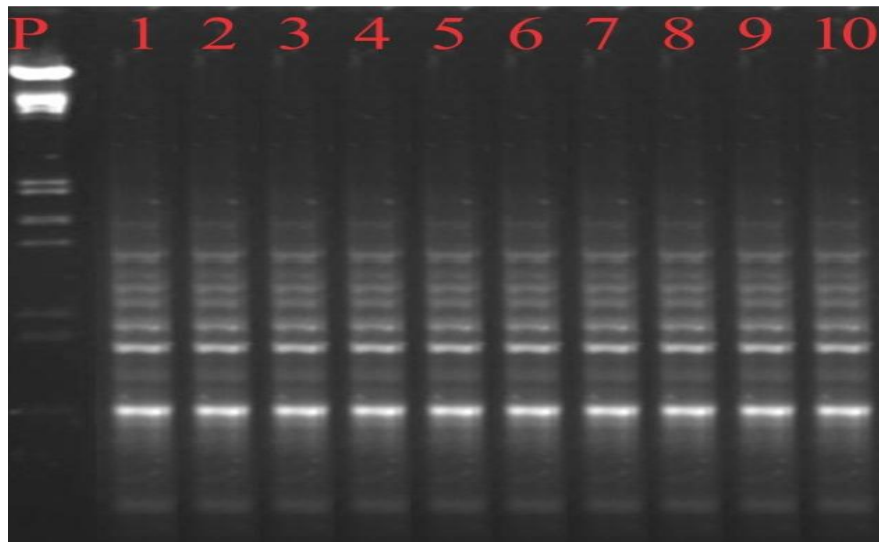


Plate 4. 1: ISSR (GACA)₄ profiles of Kenyan accessions of *L. javanica*. P- Lambda EcoRI. Accessions: Sondu (1&3), Nyahururu (2&4), Narok (5&7), Kedong (8&9) and Maralal (6&10).

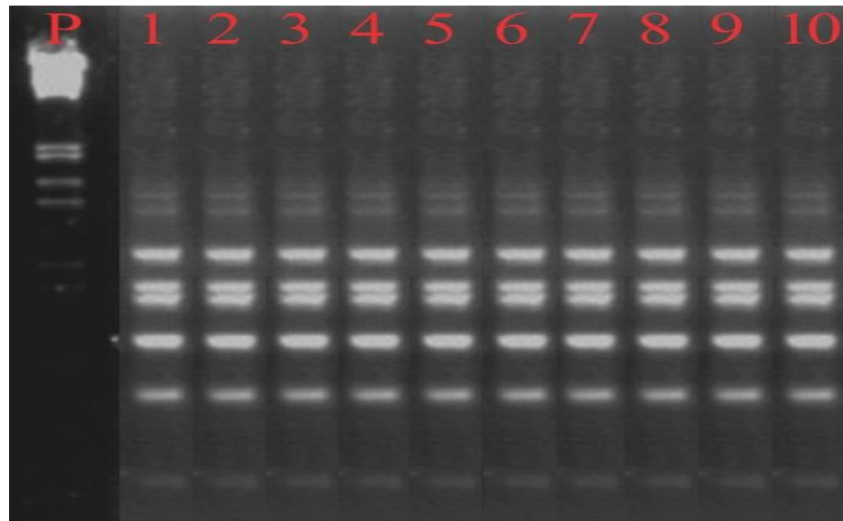


Plate 4. 2: RAPD OPA-11 profiles of Kenyan accessions of *L. javanica*. P- Lambda EcoRI. Accessions: Sondu (1&3), Nyahururu (2&4), Narok (5&7), Kedong (8&9) and Maralal (6&10).

All the bands obtained with RAPD and ISSR methods exhibited 100% monomorphism; thus the discrimination ability of the primers was zero for both the markers. The presence of only monomorphic bands indicates that the *L. javanica* complex accessions from Kenya are of one species. Identical RAPD and ISSR profiles were obtained for all the ten the plants of the *L. javanica* evaluated confirming the resemblance of the accessions (Table 4.5).

Table 4. 5: ISSR and RAPD primers used, their sequences, and band numbers produced during genetic variability analysis.

Primer	Sequence	Annealing temp (° C)	No. of Monomorphic bands	Total no. bands
ISSR 1	(AC)8T	49	2	2
ISSR 2	(GA)8Y	49	1	1
ISSR 3	(CTC)4RC	49	3	3
ISSR 4	(AG)8YC	49	2	2
ISSR 5	(GACA)4	49	3	3
ISSR 6	(CA)8G	49	1	1
			12	12
RAPD - OPZ-12	TCAACGGGAC	40	5	5
RAPD - OPZ-08	GGGTGGGTAA	40	4	4
RAPD - OPA-11	CAATCGCCGT	40	5	5
RAPD - OPZ-01	TCTGTGCCAG	40	1	1
		Total	15	15

4.3 Determination of the qualitative phytochemical compounds of *L. javanica*.

The study revealed that the extracts from the leaves of *L. javanica* had a variety of phytochemical groups (Table 4.6). Out of the 13 non-volatile phytochemical compounds screened, eleven were present. The three compound groups namely resins, phenolic glycosides, and polynuroids were confirmed to be absent in the lemon bush. Flavonoids, terpenoids, phenols, cardiac glycosides, saponins, and alkaloids were present in high amounts (++). Flavones,

anthraquinones, coumarins, anthocyanins, and tannins occurred in fairly present amounts (+) irrespective of the locality.

Table 4. 6: Results of phytochemical screening of bioactive compounds in the *L. javanica* for 10 accessions.

Samples	0019	0020	0025	0029	0035	0036	0037A	0037B	0038	0039	Observation
Test											
Alkaline reagent test	++	++	++	++	++	++	++	++	++	++	Pink scarlet color
Flavanoids	++	++	++	++	++	++	++	++	++	++	Yellow color
Flavones	+	+	+	+	+	+	+	+	+	+	Orange color
Terpenoids	++	++	++	++	++	++	++	++	++	++	Greyish color
Phenols	++	++	++	++	++	++	++	++	++	++	Blue color
Phenolic glycosides	-	-	-	-	-	-	-	-	-	-	Color persists
Cardiac glycosides	++	++	++	++	++	++	++	++	++	++	Yellow color
Saponins	++	++	++	++	++	++	++	++	++	++	Stable foam
Alkaloids	++	++	++	++	++	++	++	++	++	++	Turbidity formation
Resins	-	-	-	-	-	-	-	-	-	-	No purple color
Anthraquinones	+	+	+	+	+	+	+	+	+	+	Violet – pink color
Coumarins & anthocyanins	+	+	+	+	+	+	+	+	+	+	Pink in acidic medium
Tannins	+	+	+	+	+	+	+	+	+	+	Black color
Polynuroids	-	-	-	-	-	-	-	-	-	-	Color persists

KEY:

Sondu (0019; 0020), Kedong (0025; 0029), Nyahururu (0035; 0036), Maralal (0037A; 0037B), Narok (0038; 0039).

(++) highly present (+) fairly present, and (–) indicates absence of phytochemical.

4.4 Efficacy of the extracts of *L. javanica* in control of aphids of *V. unguiculata*.

The aphids infected all the field plots in a more or less uniform pattern after application of the five treatments. It was notable that aphid numbers increased in the plots sprayed with water and soap solution (negative control). There were significant differences in efficacy of the extracts of *L. javanica* among the treatments where the number of pests suppressed increased with increasing concentration of the treatments (Figures 4.3 and 4.4) ($F = 184.543$ and $P\text{-value} < 0.001$ Appendix 6). However, there were no significant differences between the 10% extract level and the synthetic pesticide (positive control) ($F = 184.543$ and $P\text{-value} < 0.001$) implying that the 10% concentration competed favorably with the synthetic pesticide (Atom, Osho from Syngenta).

Similarly, the severity or aphid infestation was highest where water and soap solution was applied. Aphid infestation decreased with increasing concentration of the extract. The results for analysis of variance (ANOVA) in the comparison of aphid infestation mean across the five treatments showed significant differences among the treatments ($F=194.143$ and $p\text{-value} < 0.001$) (Appendix 7). Aphid infestation was, however, least in the 10% and the synthetic pesticide (positive control), where there was no significant difference between the two treatments ($F=194.143$ and $p\text{-value} < 0.001$).

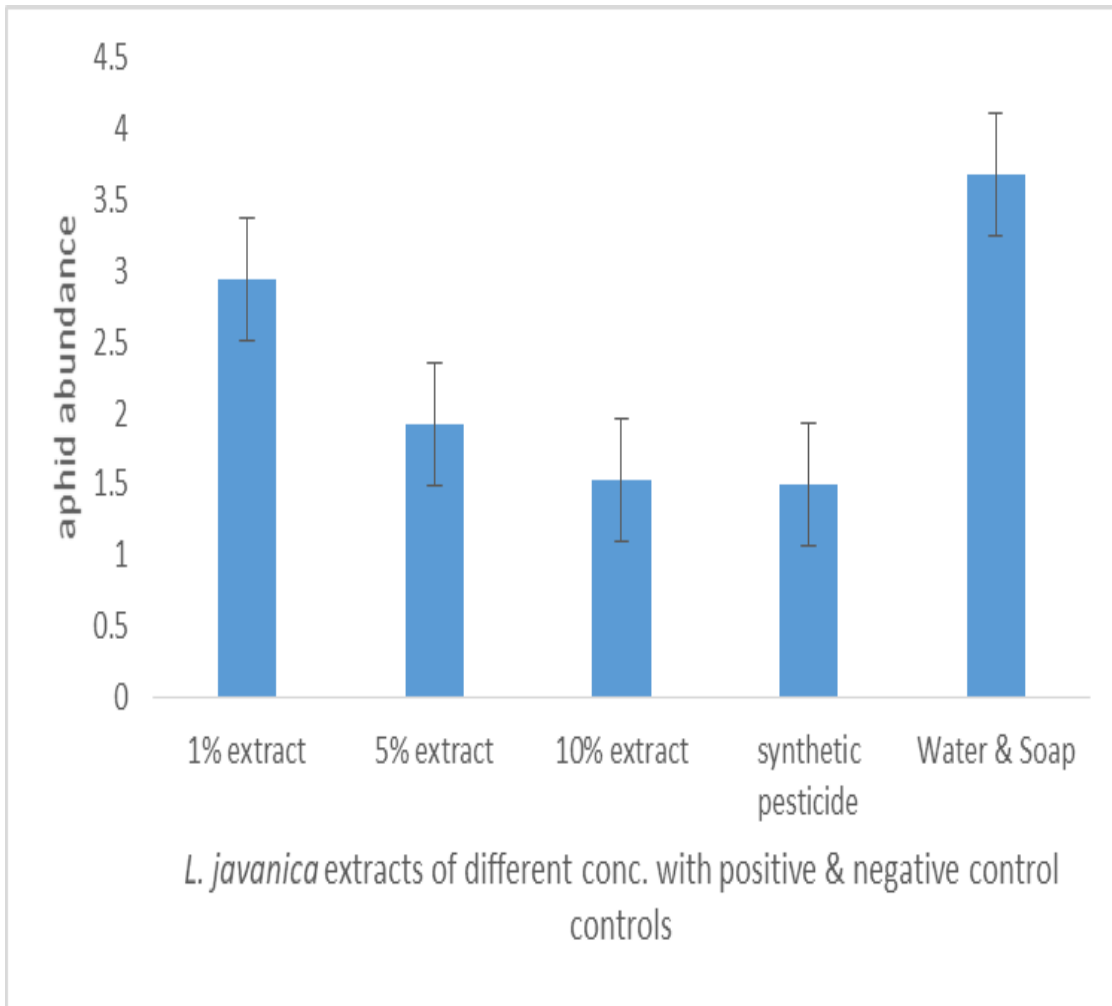


Figure 4. 3: The aphid abundance (number of aphids repelled) of cowpea aphids repelled after being sprayed with extracts of three conc. (1%, 5% & 10%) of *L. javanica* plant and positive/negative control treatments after 10 weeks of treatment.

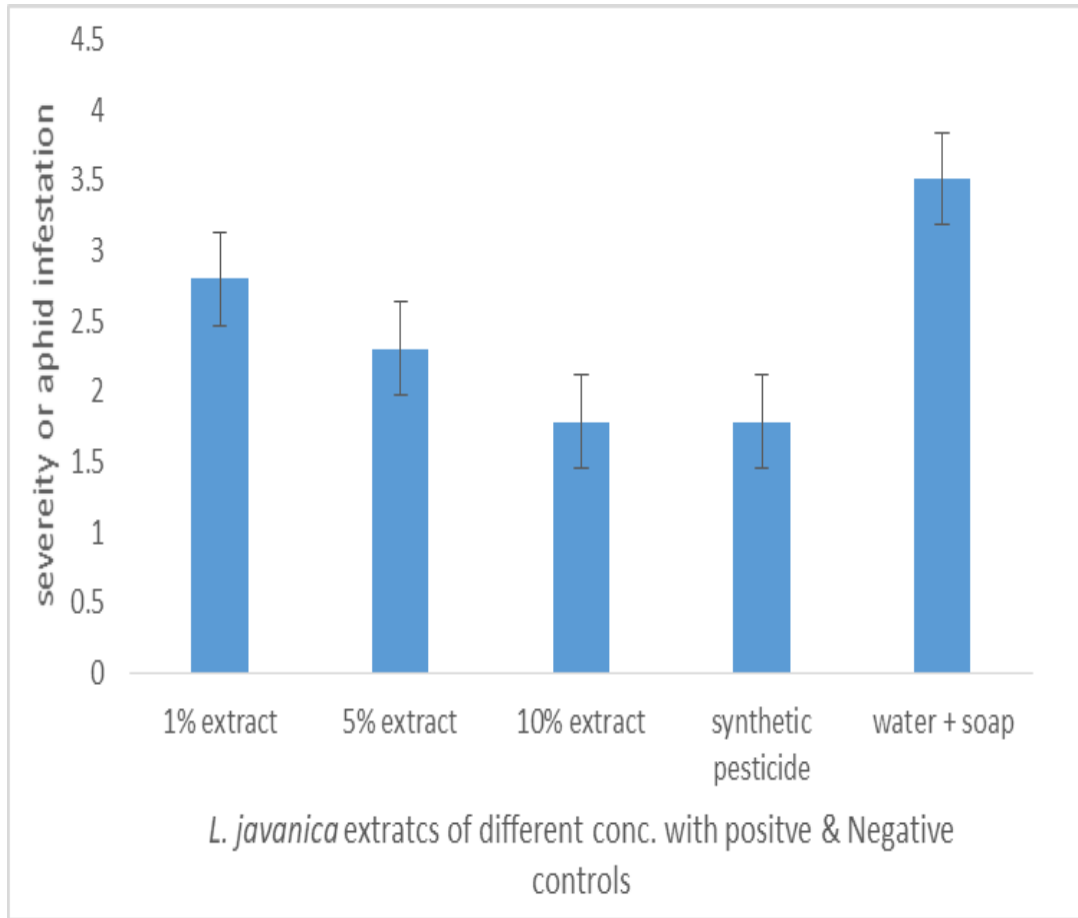


Figure 4. 4: Effect of the three conc. (1%, 5% & 10%) of *L. javanica* plant extracts and positive/negative control treatments on severity or degree of aphid infestation (damage) after 10 weeks of treatment.

CHAPTER 5: DISCUSSION

5.1 Morphological review of *L. javanica* by Cluster analysis, and Principal component analysis.

The Cluster analysis, and Principal component analysis results showed that *L. javanica* consists of variants, however, not distinct species (Figure 4.1 and 4.2) respectively. Specimens from Kedong were found to possess robust features. The peduncles were exceptionally long implying that the soil and rainfall patterns contributed to this aforementioned feature. This phenomenon is supported by factor loadings which indicate that variation in leaf sizes is what causes the clustering (Table 4.4). This implies that the difference in sizes of the *L. javanica* features is due to them occurring in different environmental places. The weak line of separation of the specimens in the cluster at 42.5 L2 dissimilarity measure might be due to environmental and altitudinal factors. According to Cron *et al.* (2006), both regional and altitudinal factors affect the morphological features of various populations, especially those at high altitudes. Rainfall patterns, temperatures, and soil types (Cron *et al.*, 2006) have been attributed to further aggravate variation. Otieno *et al.* (2006b) recorded a similar trend with the *Hemizygia bracteosa* Benth; accordingly the findings by Vwononi (2015), specimens collected from poor soils low amount of rainfall tend to have relatively smaller characters as compared to their counterparts from rich soils with even rainfall distribution. It is notable that Cron *et al.* (2007) found out a similar phenomenon in populations of *Cineraria deltoidea* Sond where some specimens had larger capitula in comparison to specimens of the same species which it was attributed to variations in soil fertility and rainfall patterns (Cron *et al.*, 2007). Sebola & Balkwill, (2013) confirms similar results in the morphological differentiation of the two forms of *Olinia rochetiana* in South Africa which seemed to reflect adaptations to the micro-climates and ecological conditions in which they occurred.

Cluster analysis has a disadvantage in that it imposes a hierarchical structure on the data and that the analysis may show distinct clusters even if the variation is clinal (morphological variation in form within a species), as may be seen using ordination techniques (Sebola & Balkwill, 2013). However, Morphometric analysis provides a powerful tool for assessing the phenetic relationships among closely related and morphologically similar taxa (Cron *et al.*, 2007). Cluster analysis (CA) based on the UPGMA method and using average taxonomic distance coefficient as a dissimilarity coefficient was used as an exploratory method to establish if the data grouped the classes (Malombe *et al.*, 2002). On the other hand, PCA is recommended for datasets containing quantitative characters and the method has been used to good effect in several taxonomic studies (Otieno *et al.*, 2006a).

The variation could also be as a result of the effects of phenetic plasticity arising from the differences in habitat. This is due to variation in characters of less taxonomic importance such as leaf characters instead of most important ones like the inflorescence. In concurrence, VWononi (2015) also reported similar occurrence of specimens of *Vernonia calvoana* to form a continuum of variation with *V. hymenolepsis*, and *V. tolypophora* in PC analysis. Verdcourt (1993) also intended to categorize *L. javanica* specimens into two varieties (*L. javanica* var. *javanica* and *L. javanica* var. *whytei*) with one being pedunculate and the other sessile. The two “varieties”, however, were not delimited either; this current study agrees with this observation.

Lippia javanica grows together in many places ranging from disturbed, scrub, and rocky hill sites as well as grasslands, open woodlands, fridges of afro-montane forests and riverbanks. In this study the concept is that a species is the smallest group that is constantly and persistently distinct, and is distinguishable by ordinary means (Amitani, 2015). The term ‘by ordinary means,’ is taken to mean the visually observable morphological differences. The existence of

gaps in the pattern, visually observable phenetic diversity is usually taken as evidence for reproductive isolation (Knowles & Carstens, 2007). In this study, the species in question lacks clear morphological discontinuities and thus not distinct. There is a compelling reason not to separate them; therefore it is proposed that *Lippia javanica* be merged into one morphologically variable species under the valid name *Lippia javanica* (Burm. F) Spreng.

5.2 Molecular characterization of *L. javanica* accessions.

The molecular characterization results show that there were no genetic variations within *L. javanica* complex accessions (Plate 4.1 and 4.2) respectively and thus *L. javanica* is one invariable species. The genotypic characters displayed no distinct polymorphism but rather similar band pattern for both ISSR and RAPD markers. It is worth noting that the molecular markers ISSR and RAPD (Table 4.5) may be trusted to efficiently identify *L. javanica* genotypes and thus allowed in the characterization. Leal *et al.* (2010) posits that using only one type of marker to quantify genetic diversity generates results that have been questioned in terms of reliability, when compared to the combined use of different markers. Similarly, Manica-Cattani *et al.* (2009) reported the efficiency of these aforementioned molecular markers for genotype identification in aromatic and medicinal plants and the characterization of *Lippia alba* has been reported. Also, ISSR markers were used to access the genetic variation in *Citrus* species where *C. volkameriana* Osbeck, *Poncirus cirumelo* Serra, and *C. trifoliata* Serra were successfully grouped into their respective categories (Salis *et al.*, 2017).

Only monomorphic bands were obtained in all the accessions by the two markers. The findings of this present study strongly suggest that *L. javanica* phenotypic differences are not supported by molecular analyses. The findings of this study are in agreement with those of Rubio-Moraga *et al.* (2009), who obtained 100% monomorphism in *Crocus sativus* L. There is congruency in the morphological and molecular results thus revealing that the accessions are

identical. This suggests that *Lippia javanica* is adapted to maintaining its genetic characteristics. Contrary to this, Manica-Cattani *et al.* (2009) reported high variation in *L. alba* from Brazil specimens. However, the percentage polymorphism was 84.1. This was expected due to the fact that they were propagated but not from natural local populations (Manica-Cattani *et al.*, 2009). In another study by Ansari *et al.* (2012), 100% polymorphism was obtained for *Tectona grandis* L.f of the central and Peninsular India regions.

The discrimination ability of the primers (Simpson Index), Mantel analysis between the Jaccard similarity coefficients, dendogram, and analysis of molecular variance (AMOVA) could not be calculated as identical RAPD and ISSR profiles were obtained for all the ten plants of the *L. javanica* complex evaluated. This confirms the resemblance of the accessions. As reported in other plant species, high correlation was observed between the distances obtained with RAPD and ISSR allowing the joint analysis of all the amplification products (Moulin *et al.*, 2012; Tamura *et al.* 2013).

5.3 Qualitative phytochemical compounds of *L. javanica*.

Lippia javanica was found to possess flavonoids, flavones, terpenoids, phenols, cardiac glycosides, saponins, alkaloids, anthraquinones, coumarines, anthocyanins, and tannins. However, polynuroids, resins, and phenolic glycosides were absent (Table 4.6) implying that the aforementioned species did not demonstrate dramatic variation in phytochemical compounds. Collection of the *L. javanica* materials in the same season and time was attributed to be the cause of lack of dramatic variation. This highly aromatic plant has been reported to display great variations in phytochemical compounds more so the essential oils (Viljoen, 2007). Furthermore, Viljoen (2007) notes that the dramatic variations in the essential and non-essential compounds might be due to regional and different harvesting time. Nevertheless, Kamanula *et al.* (2017) found out that the qualitative phytochemical compounds did not vary among the different

accessions of *L. javanica* which agrees with the results of this study. Also, essential oils have been known to vary with mycene, mycenone, linalool, caryophyllene, and p-cymene among the chemotypes isolated from *L. javanica* (Kamatou & Viljoen, 2010; Kamanula *et al.*, 2017). A study by Sahreen *et al.* (2014) found out that the non-essential greatly varied in *Rumex hastatus* L. a situation that was attributed to different harvesting time. It is worth noting that Endris *et al.* (2016a) also had similar findings in the *L. javanica* From Ethiopia. According to Nalubenga *et al.* (2014), a number of plants contain chemical components that are biologically active. These plants, therefore, have various parts such as; leaves, roots, rhizomes, stems, barks, flowers, fruits, grains or seeds, employed in the control or treatment of various disease conditions (Pochapski *et al.*, 2011). Leaves of *L. javanica* have a wide variety of the so-called classic nutrients, such as minerals, carbohydrates, proteins, fats, and vitamins (Endris *et al.*, 2016b).

According to Maroyi (2017), total phenolic compounds, tannins, and minerals have been detected in *L. javanica*. Another study by Madzimure *et al.* (2010) reports that alkaloids, flavonoids, iridoids, triterpenes, and amino acids were confirmed to occur in *L. javanica*. As a matter of fact, alanine, asparagine, and arginine were the amino acids isolated (Dlamini, 2010). Basic alkaloids, flavonoids, flavones were present in *L. javanica* (Maroyi, 2017). However, these compounds have been found to vary in quantity. *Lippia javanica* leaf extracts chemistry surprisingly varies within and between populations (Madzimure *et al.*, 2010) owing to edaphic and climatic factors (Mkenda *et al.*, 2015). This present study reaffirms results of the previous studies. While phenolic glycosides were reported in high amounts in *L. javanica* (Dlamini, 2010) found out that they were completely absent in the present study. The fluctuation in some of the compounds can be attributed to different times in harvesting, the maturity stage, and season.

5.4 Efficacy of extracts of *L. javanica* in the control of aphids of *V. unguiculata*.

This study demonstrated that *L. javanica* has pesticidal effects on aphids that affect cowpea plants (Figure 4.3). This scenario meant that the *L. javanica* possess compounds, which include alkaloids, flavonoids, flavones and terpenoids which have been reported to exhibit anti-feeding activity on insects. According to Stevenson *et al.* (2014) the botanical pesticides of *L. javanica* act in synergistic effect to deter small bodied pests including rape spider mites and aphids. Furthermore, Muzemu *et al.* (2011) states that these botanical pesticides act as anti-feedants, repellants and also as toxins. Several constituents in the volatile component have been identified in *L. javanica*. The major component camphor which have insecticidal properties (Mkenda *et al.*, 2015). Camphor occurs with minor components including camphene, α - pinene, eucalyptol, Z and E α -terpineol, linalool, cymene, thymol, 2-carene, caryophyllene and α -cubebene and may account for the biological activity of plant species in this study (Amorati *et al.*, 2013). Other potential biologically active components in *L. javanica* are mono and sesquiterpenes i.e. perialdehyde in the essential oils; this compound is highly toxic through contact with insects.

The efficacy of botanical insecticides on aphid pests was not fully effective. Some aphid pests had chances of survival perhaps through re-infection and behavioural mechanisms. Similar trends of the sought have been observed, like a study by Stevenson *et al.* (2014) found out that rape aphid pests affecting tomato developed resistance against the botanical extract of *L. javanica* and *Vernonia amygdalina*. It is also possible that the active ingredients of pesticidal plants were quickly photo degraded upon their application and therefore reducing efficacy since they are sensitive to light (Muzemu *et al.*, 2011). Dissolving of the plant powder in the water overnight might not achieve total extraction of all the pesticidal compounds. Especially non-polar compounds perhaps were not fully dissolved even though soap was used in the process. According to Mkenda *et al.* (2015) the method of extracting the active ingredients also causes

variations in concentration of the potent substance hence affect efficacy. Also, chances may be that the survival of the aphid population might be caused by new infestation from neighboring cowpea crop areas (Kamphuis *et al.*, 2012).

The extracts of *L. javanica* were able to suppress the aphids' abundance and minimize cowpea damage below their economic threshold. The reduced number of aphids and mites could be due to extracts' repellent, toxic and anti-feedant effects since they contain essential oil and alkaloids constituents with pesticidal properties (Muzemu *et al.*, 2011). These findings are in agreement with the findings of Mkenda *et al.* (2015) which found out *L. javanica* was comparable to the synthetic pesticides in reducing damage caused by foliage beetle. *Vernonia amygdalina* and *L. javanica* were the most effective plant species treatments to reduce damage caused by aphids (Mkenda *et al.*, 2015).

CHAPTER 6: CONCLUSIONS, RECOMMENDATIONS, AND SUGGESTIONS FOR FUTURE RESEARCH.

6.1 Conclusions

1. The morphological characters of *Lippia javanica* were found to be variable. Therefore, results from this study failed to validate the recognition of the proposed variants var. *javanica* and var. *whytei*.
2. The genetic characteristics of the different accessions of *L. javanica* displayed no variation using ISSR and RAPD markers confirming the proposed variants var. *javanica* and var. *whytei* as one species.
3. *Lippia javanica* was found to be rich in phytochemical compounds such as alkaloids, tannins, flavonoids, flavones, cardiac glycosides, phenols, proteins, saponins and terpenoids, which may play a vital role in repelling aphids. These compounds make the species a potential pesticidal plant.
4. There was no significant difference between the 10% extract concentration of *L. javanica* and synthetic pesticides treatments. Therefore, the extracts of the pesticidal *L. javanica* plant can be utilised as an alternative option for controlling field aphids that affect pod cowpea by smallholder farmers.

6.2 Recommendations

1. The current results have revealed that the taxonomic conflict in *L. javanica* is due to variations in non-important taxonomic characters such as the leaf sizes and robustness of the floral parts. Thus the species *L. javanica* should be treated as one.
2. *Lippia javanica* has a great potential to be used as a botanical pesticide. Its extracts should be exploited fully by smallholder farmers to repel aphids of cowpeas.

3. Numerous phytochemical compounds were detected in this aromatic medicinal plant. Phytochemists can thoroughly examine beneficial uses of the aforementioned compounds towards improving pharmaceutical industries and traditional medicine.

6.3 Suggestions for future research

1. Future molecular studies should include more markers as Simple Sequence repeats (SSRs) specific primers in combination with primers that target other abundant DNA sequences such as retrotransposons. A whole genome sequence is also needed to help discriminate the isolates of *L. javanica* complex.

2. Further studies on *L. javanica* should focus on the occurrence of new chemotypes in natural plant populations of Kenya and East Africa in an effort to reduce the impacts of aphids on cowpeas.

3. Future, studies should investigate on the specific phytochemical components responsible for pesticidal efficacy in *L. javanica*.

4. Future scientists should employ different techniques of extracting the botanical extracts of the *L. javanica* for instance use of ethanol and warm water to maximize the extraction of polar and non-polar compounds.

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Appendices

Appendix 1: List of *L. javanica* specimens examined for extraction of morphological data.

Collectors No	Species	Country	Locality	FTEA	Latitude	Collection date
1007	<i>L. javanica</i>	U	Bufumbiro	U3	1°12' S,	11/01/1932
433	<i>L. javanica</i>	U	Kapchorwa	U3	29°40' E	12/10/1952
712	<i>L. javanica</i>	T	Rufiji	T8	1°24' N,	10/08/1969
11049	<i>L. javanica</i>	T	Nyamabamyito	T7	34°27' E	11/07/1956
9397	<i>L. javanica</i>	T	Songea	T8	8°19' S,	02/04/1956
327	<i>L. javanica</i>	T	Mbulu	T2	38°10' E	05/09/1962
4422	<i>L. javanica</i>	T	Momela	T2	9°19' S,	15/11/1964
5443	<i>L. javanica</i>	T	Musoma	T1	34°46' E	08/12/1970
10686	<i>L. javanica</i>	T	Maswa	T1	10°41' S,	04/06/1962
7749	<i>L. javanica</i>	T	Hanang Mt.	T2	35°43' E	14/02/1946
12614	<i>L. javanica</i>	T	Masai	T2	3°30' S,	24/07/1966
002	<i>L. javanica</i>	T	Mweka	T2	35°45' E	03/11/1966
10684	<i>L. javanica</i>	T	Songea	T8	3°15' S,	01/03/1956
10189	<i>L. javanica</i>	T	Mbeya	T7	36°51' E	12/05/1956
6538	<i>L. javanica</i>	T	Ndunduru FR	T7	2°20' S,	23/09/2000
10979	<i>L. javanica</i>	T	Lukumburu	T2	34°50' E	06/07/1956
525	<i>L. javanica</i>	T	Iringa	T7	2°20' S,	25/03/1976
11064	<i>L. javanica</i>	T	Mufindi	T7	34°50' E	13/08/1971
7014	<i>L. javanica</i>	T	Mpwapwa	T5	3°00' S,	15/07/1950

29128	<i>L. javanica</i>	T	Bereku	T5	35°29' E	01/04/1974
405	<i>L. javanica</i>	T	Ruaha River	T6	3°00' S,	13/07/1970
10147	<i>L. javanica</i>	T	Gumbiro	T8	35°29' E	10/05/1956
1098	<i>L. javanica</i>	T	Lyamungu	T2	3°14' S,	27/10/1943
182	<i>L. javanica</i>	T	Nyiro	T6	37°19' E	01/06/1954
52	<i>L. javanica</i>	K	Olorgorot P	K6	10°41' S,	31/07/1968
1622	<i>L. javanica</i>	K	Nakuru	K3	35°39'E	25/07/1962
726	<i>L. javanica</i>	K	L. Naivasha	K5	8°50' S,	18/04/1968
973	<i>L. javanica</i>	K	Sotik	K5	33°20' E	15/06/1953
80	<i>L. javanica</i>	K	Marakwet	K2	7°47' S,	01/09/1957
382	<i>L. javanica</i>	K	Muthangari	K4	36°28' E	08/08/1977
756	<i>L. javanica</i>	K	L. Naivasha	K5	9°44' S,	19/04/1968
2413	<i>L. javanica</i>	K	Fort Hall	K4	35°09' E	04/08/1968
568	<i>L. javanica</i>	K	Kanzui area	K4	8°35' S,	13/08/1968
003	<i>L. javanica</i>	K	Mai Hahiu	K4	35°15' E	09/06/2001
1059	<i>L. javanica</i>	K	Mount Elgon	K3	8°35' S,	26/05/1948
6037	<i>L. javanica</i>	K	Narok	K6	35°15' E	19/05/1977
2873	<i>L. javanica</i>	K	Kipleleo	K6	6°30' S,	24/05/1962
3124	<i>L. javanica</i>	K	Olekaitorror	K6	36°29' E	14/07/1962
2033	<i>L. javanica</i>	K	Ol-ari-nyiro R	K3	4°27' S,	23/07/1988
247	<i>L. javanica</i>	K	Maralal	K1	35°44' E	01/11/1976
49	<i>L. javanica</i>	K	Ngongogeri F	K3	6°20' S,	14/08/1931
529	<i>L. javanica</i>	K	Mathews R	K1	37°00' E	19/05/1958

127	<i>L. javanica</i>	K	Kipkaren	K3	10°16' S,	28/06/1985
304	<i>L. javanica</i>	K	SW Elgon	K5	35°39'E	10/09/1965
188	<i>L. javanica</i>	K	Kitale	K3	3°14' S,	18/02/1970
11	<i>L. javanica</i>	K	Njoro	K3	37°15' E	30/03/1977
2517	<i>L. javanica</i>	K	Kedong R	K3	6°20' S,	21/05/1976
263	<i>L. javanica</i>	K	Masai Mara	K6	37°00' E	19/10/1971
217	<i>L. javanica</i>	K	Narok	K6	0°43' S,	04/09/1965
1907	<i>L. javanica</i>	K	Mathere V	K4	36°26' E	02/11/1984
355	<i>L. javanica</i>	K	Muguga	K4	0°15' S,	22/08/1992
284	<i>L. javanica</i>	K	Kiminini	K3	35°54' E	25/06/2003
3283	<i>L. javanica</i>	K	Chyulu Hills	K4	0°45' S,	21/04/1938
28	<i>L. javanica</i>	K	Kuraiha F	K4	36°21' E	01/08/1957
253	<i>L. javanica</i>	K	Chyulu Hills	K4	0°41' S,	25/05/1936
1014	<i>L. javanica</i>	K	Sotik	K5	35°06' E	23/08/1975
22	<i>L. javanica</i>	K	Kabete	K4	0°49' N,	13/12/1960
3734	<i>L. javanica</i>	K	Songhor	K5	35°36' E	01/04/1961
004	<i>L. javanica</i>	K	Narok	K6	1°17' S,	13/12/1967
330	<i>L. javanica</i>	K	Masai Mara	K6	36°49' E	01/04/1961
9356	<i>L. javanica</i>	K	Ngoina	K5		13/12/1967
1622	<i>L. javanica</i>	K	Nakuru	K3		25/07/1962

Appendix 2: A herbarium voucher specimen displaying *L. javanica*.

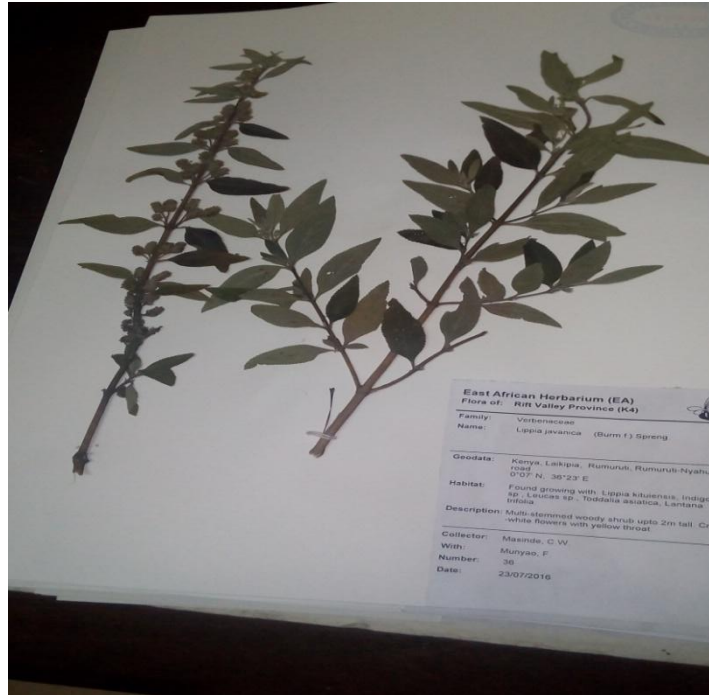


Photo by: Collins January, 12th 2017

Appendix 3: UV trans-illuminator in the laboratory used for observation of the gel.



Photo by: Collins November 23rd 2016

Appendix 4: Band scoring matrix from the PCR agarose gel for RAPD markers for the 10 *L. javanica* accessions; (1) indicating band presence and (0) for absence.

Band	Accessions									
	Sond u	Nyahurur u	Sond u	Nyahurur u	Naro k	Maral al	Naro k	Kedon g	Kedon g	Maral al
B1	0	0	0	0	0	0	0	0	0	0
B2	0	0	0	0	0	0	0	0	0	0
B3	0	0	0	0	0	0	0	0	0	0
B4	1	1	1	1	1	1	1	1	1	1
B5	1	1	1	1	1	1	1	1	1	1
B6	1	1	1	1	1	1	1	1	1	1
B7	1	1	1	1	1	1	1	1	1	1

Appendix 5: Biochemical screening test for cardiac glycosides. The second photo showing lower layer as blue green which is a confirmatory test for cardiac glycosides



Photo by: Collins November 2rd, 2016

Appendix 6: ANOVA table for comparing the aphids' abundance across all treatments.

	<i>Sum of Squares</i>	<i>df</i>	<i>Mean Square</i>	<i>F</i>	<i>Sig.</i>
<i>Between Groups</i>	742.844	4	185.711	184.543	.000
<i>Within Groups</i>	1001.300	995	1.006		
<i>Total</i>	1744.144	999			

Appendix 7: ANOVA table for comparing the severity or degree of infestation.

	<i>Sum of Squares</i>	<i>df</i>	<i>Mean Square</i>	<i>F</i>	<i>Sig.</i>
<i>Between Groups</i>	432.296	4	108.074	194.153	.000
<i>Within Groups</i>	553.860	995	.557		
<i>Total</i>	986.156	999			

Appendix 8: The results for Least Significant Difference (LSD) Test.

(I) TRT	(J) TRT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
T1	T2	-.495*	.075	.000	-.64	-.35
	T3	.520*	.075	.000	.37	.67
	T4	.520*	.075	.000	.37	.67
	T5	-1.210*	.075	.000	-1.36	-1.06
T2	T1	.495*	.075	.000	.35	.64
	T3	1.015*	.075	.000	.87	1.16
	T4	1.015*	.075	.000	.87	1.16
	T5	-.715*	.075	.000	-.86	-.57
T3	T1	-.520*	.075	.000	-.67	-.37
	T2	-1.015*	.075	.000	-1.16	-.87
	T4	.000	.075	1.000	-.15	.15
	T5	-1.730*	.075	.000	-1.88	-1.58
T4	T1	-.520*	.075	.000	-.67	-.37
	T2	-1.015*	.075	.000	-1.16	-.87
	T3	.000	.075	1.000	-.15	.15
	T5	-1.730*	.075	.000	-1.88	-1.58
T5	T1	1.210*	.075	.000	1.06	1.36
	T2	.715*	.075	.000	.57	.86
	T3	1.730*	.075	.000	1.58	1.88
	T4	1.730*	.075	.000	1.58	1.88

KEY:

*. The mean difference is significant at the 0.05 level.

T1- 5% conc. Extract of *L. javanica*

T2- 10% conc. Extract of *L. javanica*

T3-1% conc. Extract of *L. javanica*

T4-synthetic pesticide (positive control).

T5- Water plus soap solution (Negative control).