MOLECULAR PHYLOGENY, LEAF MICROMORPHOLOGY AND ANTIMICROBIAL ACTIVITY OF PHYTOCONSTITUENTS OF KENYAN *Plectranthus* SPECIES IN THE *COLEUS* CLADE

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DECLARATION

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

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DEDICATION

This thesis is dedicated to my family and friends who provided me with moral and financial support throughout my studies.

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LIST OF ABBREVIATIONS AND ACRONYMS

^{13}C NMR:	13 Carbon Nuclear Magnetic Resonance
^{1}H NMR:	1 Hydrogen Nuclear Magnetic Resonance
ATCC:	American Type Culture Collection
BLAST:	Basic Local Alignment Search Tool
CC:	Column Chromatography
$CDCL_3$:	Deuterated Chloroform (Chloroform D).
COSY:	Homonuclear correlation spectroscopy
DCM:	Dichloromethane
DMSO:	Dimethylsulphoxide
EDTA:	Ethylenediaminetetraacetic acid
ESTOFMS	Electron Spray Time of Flight Mass Spectroscopy
EtoAC:	Ethyl Acetate
EtOH:	Ethanol
FTEA:	Flora of Tropical East Africa
HMBC:	Heteronuclear Multiple Bond Correlation
HSQC:	Heteronuclear Single Quantum Correlation
LCC:	Liquid Column Chromatography
MatK:	Maturase K gene
MEGA 6:	Molecular Evolutionary Genetics Analysis Version 6
MeOH:	Methanol
MIC:	Minimum Inhibitory Concentration
MRSA:	Methicillin Resistant *Staphylococcus aureus*
MS:	Mass spectroscopy
NCBI:	National Center for Biotechnology Information
OTU:	Operational Taxonomic Units
PLC:	Partition Liquid Chromatography
Rbcl:	Large subunit of the ribulose-bisphosphate carboxylase gene
SPSS 23:	Statistical Package for the Social Sciences Version 23
TLC:	Thin Layer Chromatography
UPGMA:	Unweighted Pair Group Method with Arithmetic Mean

ABSTRACT

Plectranthus L'Hér. is an economically important genus with horticultural, medicinal and food uses. Several *Plectranthus* species are used in traditional medicine and have attracted the interest of researchers who have studied them in attempt to explore their bioactive compounds. Despite the wide usage of the genus, *Plectranthus* species are difficult to taxonomically delimit due to lack of clear cut morphological synapomophies. This study aimed at bringing insights into *Plectranthus* classification by incorporating additional criteria from stomatal distribution, leaf anatomy and molecular phylogeny using *Rbcl* and *MatK* DNA base sequences.The study also investigated the phytoconstituents and antimicrobial activities of *Plectranthus* species. Stomatal density and distribution was determined by stomatal counts on the abaxial and adaxial leaf surfaces while leaf anatomical structures were observed in microtome-leaf sections. Bioassay guided fractionation was used to isolate bioactive compounds while antimicrobial activity was determined though broth dilution and disc diffusion methods. Genomic DNA from each species was extracted followed by amplification and sequencing of the *MatK* and *Rbcl* genes. *MatK* and *Rbcl* gene sequences were aligned and analyzed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Bootstrap resampling in Molecular Evolutionary Genetics Analysis Version 6 (MEGA6) to generate phylogenetic trees. Leaf micromorphology data was analyzed through cluster analysis while antimicrobial data was analyzed by ANOVA in SPSS. Results of the molecular phylogeny based on the *MatK* and *Rbcl* gene sequences clustered four species *Plectranthus caninus* Roth., *Plectranthus otostegioides* (Gürke) Ryding, *Plectranthus barbatus* Andrews and *Plectranthus lanuginosus* (Hochst. ex Benth.) Agnew together: Clusters A and D respectively while *Plectranthus pseudomarrubioides* Willemse, *Plectranthus ornatus* Codd., *Plectranthus aegyptiacus* (Forssk) C.Chr.were grouped together into Clusters B and E respectively while *Plectranthus montanous* Benth. and *Plectranthus amboinicus* (Lour.) Spreng were grouped together (Cluster C). From the leaf micromorphology study, epidermal cell type, trichome type, nature of the leaf mesophyll and the palisade layer contributed to most of the leaf anatomical differences among the ten *Plectranthus* species. A dendrogram generated through cluster analysis of the leaf micromorphological characters grouped together *P.caninus, P.ornatus, P. otostegioides, P.montanous* and *P. pseudomarrubioides* (Cluster F). The dendrogram also grouped together *P. aegyptiacus, P. amboinicus, P. edulis, P. barbatus* and *P. lanuginosus* (Cluster G). Upon determination of antimicrobial activity of Dichloromethane: Methanol (1:1) leaf crude extracts from the ten *Plectranthus* species, *P. barbatus*, *P. lanuginosus* and *P.ornatus* leaf crude extracts inhibited the growth of microbes better compared to the other *Plectranthus* species under investigation. At a concentration of 200 mg/ml, crude leaf extracts of *P. barbatus* and *P. lanuginosus* growth inhibitions against methicillin resistant *Staphylococcus aureus* (MRSA) were not significantly different from the growth inhibition of 50 mg/ml of Amoxicillin against MRSA at $p \leq 0.05$. In addition, at a concentration of 200 mg/ml, total leaf extracts from *P.ornatus* and *P. barbatus* leaf crude extracts growth inhibitions against *Candida albicans* were not significantly different from growth inhibition of 40 mg/ml of Ketoconazole against *C.albicans* $p \leq 0.05$. Dichloromethane: Methanol (1:1) total leaf extracts from *P. barbatus* displayed higher growth

inhibitions against the test microbes compared to the other nine *Plectranthus* species with MIC values of 25 mg/ml, 40 mg/ml, 100 mg/ml, 50 mg/ml and 100 mg/ml against MRSA, *Bacillus cereus, Escherichia coli, C.albicans* and *Aspergillus niger* respectively. Bioassay guided fractionation of the DCM total extracts from the leaves of *P.barbatus* led to the isolation and purification of two new abietane diterpenes; Compound A and B from the dichloromethane partition. Compound A exhibited MIC values of 40 mg/ml, 40 mg/ml, 50 mg/ml, 50 mg/ml and 40 mg/ml against MRSA, *B.cereus, E.coli*, C.*albicans* and *A.niger*, respectively. The MIC values observed from compound B were 20 mg/ml, 50 mg/ml, 20 mg/ml, 40 mg/ml and 50 mg/ml against MRSA, *B.cereus, E.coli*, C.*albicans* and *A.niger*, respectively. These two compounds were simply referred to as (16S)-MethoxyColeon E (Compound A) and (16R)-MethoxyColeon E (Compound B) due to their similarity to Coleon E. Their antimicrobial activity against the test microbes was considered low or negligible because their MIC values were above 100 µg/ml which ranged from 20 mg/ml to 50 mg/ml. The current study also reports for the first time, the antimicrobial activity of *P. pseudomarrubioides, P.edulis, P. aegyptiacus, P. otostegioides* and *P. lanuginosus*. In addition, the study has demonstrated broad bacteriostatic activity of *P. barbatus* and thus recommends further studies aimed at discovery of novel antimicrobial ligands. With regard to phylogeny, the present study has grouped the ten study *Plectranthus* species using molecular and leaf micromorphology characters into phylogenies which are supported by previous studies and proved that molecular and micromorphology characters can aid in plant identification and phylogenetic studies.

Key words: *Plectranthus*; leaf micromorphology; molecular phylogenetics, antimicrobial activity, bioactive compounds, MIC, Bioguidance

CHAPTER ONE: INTRODUCTION

1.1. Background information

Plectranthus is a significant and extensively used genus in many parts of the world and its use range from medicinal and food uses to horticultural uses (Rice *et al*., 2011). With respect to its medicinal use, many species of *Plectranthus* have been used to treat a wide variety of diseases in traditional medicine (Gaspar-Marques *et al*., 2006). Ethnobotanical usage of *Plectranthus* has led to more research on specific species of *Plectranthus* leading to isolation of various bioactive compounds such as terpenes, flavonoids and essential oils with various bioactivities (Paton *et al.*, 2004; Dellar *et al.*, 1996). Such studies have proved the importance of *Plectranthus* species in drug research and development (Rice *et al*., 2011).

With respect to its horticultural value, *Plectranthus* species such as *Plectranthus oertendahlii* T.C.E.Fr. and *Plectranthus parvifolius* (Batalin) C. P'ei make good ground covers since many species thrive in large numbers (Rice *et al*., 2011). *P. oertendahlii* and *P. parvifolius* can be grown from cuttings and seeds, they can grow on hanging baskets and require little maintenance practices (Rice *et al.*, 2011). *Plectranthus* species are attractive and floriferous due to their purplish flowers and their salient cultural advantages include low light requirement, adaptation to semi-arid conditions and tolerance to warm dry atmosphere (Rice *et al.*, 2011). *Plectranthus* species such as *P. amboinicus*, *Plectranthus esculentus* N.E.Br., *P. edulis* and *P. barbatus* are also used as fodder crops, food additives as well as flavors in many parts of the world due to their aromatic nature (Rice *et al*., 2011).

Despite the fact that *Plectranthus* have a wide variety of uses, difficulties exist in its classification. For instance *Plectranthus* genus is characterized by a lot of synonymy whereby the

same plant is referred by different names (Lukhoba *et al*., 2006). Lack of clear morphological synapormophies has made it hard to delimit taxa within *Plectranthus* (Grayer *et al*., 2010). Initially *Plectranthus* were classified by use of single characters only and recently molecular characters and biochemical characters have been employed to clarify the original classification based on morphological characters. For example, Paton *et al.* (2004) successfully used molecular characters to classify members of Labiatae family, while Grayer *et al.* (2010) studied the distribution of exudate flavonoids in *Plectranthus* to bring light into its classification.

For better understanding of the relationships among *Plectranthus* species, a comprehensive phylogenetic study needs to be conducted on this genus to refine taxonomic and evolutionary relationships utilizing different types of data such as morphology, biochemical, cytology, anatomy and molecular data. As a result of these taxonomic inconsistencies, this study focused on molecular phylogeny of *Plectranthus,* leaf anatomy and leaf stomatal distribution (leaf micromorphology) of the Kenyan *Plectranthus* species to better understand the taxonomic relationships of Kenyan *Plectranthus* species in the *Coleus* clade. The study also investigated the antimicrobial activity of the Kenyan *Plectranthus* species since they have been used widely in traditional medicine in the treatment of various ailments and are assumed to contain important phytoconstituents (Lukhoba *et al.,* 2006). Data obtained was compared with the existing data on *Plectranthus* classification to bring about clarity on the relationships among ten *Plectranthus* species belonging to the *Coleus* clade.

1.2. Economic importance of *Plectranthus*

Plectranthus species are commonly known as spur flowers. They are grown and extensively used in various parts of the world as ornamentals, leafy vegetables, root vegetables due to their edible tubers and have numerous medicinal uses (Lukhoba *et al.*, 2006). About 85 %

of all species of *Plectranthus* known to man have been reported to have medicinal value and *Plectranthus* is mostly cited in literature for its medicinal uses (Lukhoba *et al.*, 2006). Majority of species within *Plectranthus* are used in folk medicine to treat a variety of diseases including infectious conditions (Matu and van Staden, 2003) and the potential medicinal and economic use of *Plectranthus* are of great interest (Grasper-Marques *et al.,* 2006). Stems, leaves, tubers of different *Plectranthus* species are used to treat a variety of diseases ranging from bacterial, fungal to viral diseases (Cook, 1995; Hutchings *et al.*, 1996). The wide variety of ailments treated by species within *Plectranthus* is an indication of the medicinal value of the genus and the scope of drug development in this genus is endless (Rice *et al*., 2011).

Plectranthus has valuable bioactive compounds with the aromatic nature of the genus being attributed to essential oil production. Saponins, monoterpenes, sesquiterpenes, diterpenes and phenolic compounds have been isolated and reported in many species of *Plectranthus* (Abdel-Mogib *et al*., 2002; Gaspar-Marques *et al*., 2004). Phytoconstituents such as diterpenes from *Plectranthus* have been reported to possess myriad of bioactivities (Dellar *et al.*, 1996). Bioactivities reported from *Plectranthus* species include; antibacterial and antifungal (Simoes *et al.* 2010), antiplasmodial (Van-Zyl *et al.*, 2008), anti-cancer (Marques *et al*., 2002) and insect repellent activity (Grayer *et al.*, 2010). Other reported bioactivities from *Plectranthus* species include: antioxidant activity, ovicidal, antimalarial, antiviral, antileishmanial, larvicidal, insect antifeedant and anti-inflammatory activities besides others (Dhukhea, 2010). These pharmacological activities of species within *Plectranthus* make them important in natural product research.

Plectranthus is a genus with a variety of horticultural uses. *Plectranthus* has been described as genus of horticultural importance by Van Jaarsveld (2006). *Plectranthus* genus has attracted the

interest of many horticulturists who have embarked on breeding *Plectranthus* in order to come up with novel flowers for the horticultural industry (Rice *et al.*, 2011). Species such as *Plectranthus oertendahlii* T.C.E.Fr. and *P. parvifolius* are grown in hanging baskets, pots and containers. Indeed *P. oertendahlii* (Swedish ivy) has been used as a horticultural crop for over 100 years (Rice *et al.*, 2011). *Plectranthus* is a horticulturally important genus consisting mostly herbaceous plants which are widely used in indigenous landscaping, as hedges, ground covers and act as good species for rockeries and succulent gardens. (Rice *at al*., 2011). Other species are used as food additives and fodder crops such as *P. amboinicus*, *P. barbatus* and *P. esculentus* N.E.Br. Herbaceous *Plectranthus* form a fairly new resource in continued exploitation of wildflower diversity and have been bred to utilize this remarkable diversity with amenity horticultural potential (Rice *et al.* 2011). *Plectranthus* has a wealth of ethnobotanical species which forms a basis for research in both horticultural and in the health sector.

1.3. A taxonomic review of *Plectranthus*

Plectranthus belongs to the family Labiatae (Nepetoideae, tribe Ocimeae). *Plectranthus* genus consists of about 300 species distributed in the tropical and warm regions of the world (Rice *et al.*, 2011). *Plectranthus* species are aromatic, perennial sub-shrub or succulent shrub ranging from 0.2 m to 5 m tall. They are succulent herbs, fleshy and highly aromatic, much branched, possessing short soft erect hairs, with distinctive smelling leaves. The stem four angled, fleshy either with long rigid hairs or densely covered with soft, short and erect hairs. Leaves are simple, broad, oval-shaped with a tapering tip and very thick; they are thickly studded with hairs and the lower surface usually has the most numerous glandular hairs, giving a frosted appearance. The taste of the leaves is pleasantly aromatic with agreeable and refreshing odour. Flowers are borne

on a short stem, pale purplish in dense whorls at distant intervals in a long slender raceme (Paton *et al*., 2009).

Two clades have been identified in *Plectranthus* L'Her: *Plectranthus* clade and *Coleus* Lour clade. Many species formerly classified in the genus *Coleus* have now been placed in *Plectranthus.* Initially the two genera had been separated on the basis of gross morphological characters only (Grayer *et al*., 2010). In addition several other closely related genera within Labiatae such as *Ocimum* L., *Solenostemon* Thonn. and *Englerastrum* Briq. are still treated separately and therefore *Plectranthus* in broad sense could be still paraphyletic (Grayer *et al.*, 2010). It has proved difficult to classify species within *Plectranthus* genus as there are no clear morphological synapomorphies. Furthermore, the genus is faced with a lot of synonymy whereby a species is referred by another name somewhere else (Lukhoba *et al.*, 2006).

1.4. Taxonomic difficulties in *Plectranthus* genus

Taxonomic relationships among *Plectranthus* are not well understood. Few taxonomic studies have been published on phylogenetic relationships among *Plectranthus* species. Initially, *Plectranthus* species were classified using morphological characters only and this led to placement of various species into a different family such as Verbenaceae rather than Labiatae family (Paton *et al*., 2004). In the latest revision of the genus based on morphological characters, two major clades are recognized within *Plectranthus* genus: *Plectranthus* and *Coleus* clades (Paton *et al.*, 2009). *Plectranthus* clade consists of about 30 species while *Coleus* clade consists of 70 species. Paton *et al*., (2009) further divided the *Coleus* clade into three sub-clades which included *Holostylon* (species with an entire style), *Solenostemon* (species with fused anterior calyx lobes) and *Calceolanthus* (species whose calyx throat is surrounded by dense ring of hairs). Unlike *Plectranthus* clade, the most taxonomic difficulties are observed in the *Coleus* clade which is well

represented in Kenya and of which majority of the species are used as medicinal plants (Lukhoba *et al.,* 2006).

Paton *et al.* (2004) proposed a new classification system using molecular data where he carried out a phylogeny of basils and allies (Labiatae, tribe Ocimeae) based on sequences of the *trnL* intron, *trnL-trnF* intergene spacer and *rps16* intron for better understanding of the relationships among members of the tribe Ocimeae which included *Plectranthus*. Paton *et al*. (2004) concluded that the Tribe Ocimeae was monophyletic and easily diagnosable with morphological synapomorphies. Lukhoba *et al.* (2006) mapped *Plectranthus* economic uses to the phylogenetic tree based on molecular analysis and concluded that species within the *Coleus* clade were richer in number and diversity of uses among the species of *Plectranthus*. Grayer *et al.* (2010) carried out chemosystematic study by looking at the distribution of exudate flavonoids in *Plectranthus*. *Plectranthus* still remains a controversial genus when it comes to classification and some species grouped under other genera could still be under *Plectranthus*. Grayer *et al*. (2010) found out that 40% of the species within the *Plectranthus* clade produced exudate flavonoids mainly flavones and that at least five species within the *Plectranthus* clade could produce flavanones while flavanols were only found in two species within the *Coleus* clade.

Stomatal distribution and leaf anatomy may provide insights into how *Plectranthus* species evolve in terms of leaf anatomy and respond to different climatic conditions. Leaf anatomy and tomentum morphology of leaves and calyces of six species of *Cyclotrichium* (Boiss.) Manden. & Scheng. (Labiatae) in Turkey were used as taxonomic characters which lead to a better understanding of the relationships of the six species (Satil *et al*., 2011). Elsewhere morphology of epidermal cells, stomata and trichome types have been used as systematic characters and a combination of some of these features are relevant, especially for the identification of species

within the tribe Mentheae (sub-family Nepetoideae) in Labiatae family (Moon *et al*., 2009). Davis and Barnett, (1997) used leaf anatomical features such as arrangement of the epidermal cells, air cavities, vascular bundles, presence of wax, number of stomata, nature of the spongy mesophyll layer and palisade layers to come up with data which can be used to group taxonomically difficult species and subspecies of *Galanthus* L. genus (Amaryllidaceae). Elsewhere, morphology of trichomes has been used in infrageneric classification of *Stachys*, *Teucrium* and *Chelonopsis* (Labiatae) (Salmaki *et al*., 2009) while trichome morphology studies have also been done on species of the genus *Salvia* in the family Labiatae (Bisio *et al*., 1999).

More studies are needed to investigate whether *Plectranthus* is monophyletic, to test the current generic circumscription and to determine morphological characters that are suitable to support a comprehensive phylogeny based classification. For example, studies on molecular phylogeny of *Plectranthus* using sequences from various regions of nucleus and chloroplast DNA such as *trnK*, nuclear ITS, *Rbcl* and *MatK or* even using other methods such as SNPs (Single Nucleotide Polymorphisms), RAPDS (Random Amplified Polymorphic DNA) and RFLPs (Restriction Fragment Length Polymorphisms) need to be conducted. These molecular characters and methods may suggest relationships in phylogeny as they have been used successfully in other species (Catalan *et al*., 1995; Abdulla and Gamal, 2010; Hajibabaei *et al*., 2007). Studies on the distribution of various phytochemicals, leaf micromorphology and cytology are also needed and may help in phylogenetic elucidation of *Plectranthus* genus. This will help in the development of a formal classification since the current groupings of *Plectranthus* species are not congruent with previous morphological classification of *Plectranthus* species (Paton *et al.*, 2009).

1.5. Problem Statement

Plant species should have only one name and should be easily identified from other related species based on their unique diagnostic taxonomic characters. However, most Kenyan *Plectranthus* species have similar morphological characters and it is difficult to differentiate individual species based on such morphological characters since there are no clear morphological synapormophies (Paton *et al*., 2004). In addition, various species have been given several different names and hence this phenomenon of synonymy within *Plectranthus* genus has also made it difficult to place *Plectranthus* species in their right classification positions and collate together the uses of specific species (Lukhoba *et al*., 2006). *Plectranthus* species have been used widely in traditional medicine for treatment of various diseases ranging from bacterial, viral and fungal infections to conditions such as pains and headaches (Rice *et al*., 2011). *Plectranthus* species are rich in bioactive terpenoids and phenolic compounds. The current study therefore determined whether leaf anatomical characters and molecular phylogeny using *Rbcl* and *MatK* DNA sequences may be used in the delimitation of the Kenyan *Plectranthus* species. Determination of antimicrobial activity and identification of bioactive compounds through bioassay guided fractionation of the Kenyan *Plectranthus* species will allow researchers to identify potential therapeutic applications of *Plectranthus* even though they may have no documented activity.

1.6. Justification

Few global taxonomic studies have been published on *Plectranthus* genus (Grayer *et al*., 2010). A comprehensive revision utilizing different types of data including morphological, biochemical, anatomical and molecular data is needed. Molecular characters are not influenced by the environment like morphological characters. In addition, anatomical and molecular characters are more diagnostic in value and can help in identification of species which are hard to delimit

using morphological characters. With molecular characters, homology assessment is easy and one can easily determine monophyletic groups from the phylogenetic trees produced by molecular characters. Classification of *Plectranthus* species based on anatomical and molecular characters will improve the existing classification and provide a framework and guidelines for plant improvement and help to preserve genetic resources of *Plectranthus* species. Species within *Plectranthus* genus have many uses, such as medicinal, leafy and root vegetables and as horticultural crops. Moreover carrying out a bioassay guided fractionation of *Plectranthus* can help in the identification of compounds with useful bioactivities from this genus which can act as lead compounds in drug development against sensitive and resistant pathogenic microbes.

1.7. Hypotheses

H_O: There are no differences in leaf anatomy and stomatal distribution among the ten *Plectranthus* species in the *Coleus* clade investigated.

H_O: Maturase K and ribulose-bisphosphate carboxylase (*Rbcl*) gene sequences do not differ among the ten *Plectranthus* species in the *Coleus* clade investigated.

H_O: The ten *Plectranthus* species in the *Coleus* clade investigated have no antimicrobial activity.

1.8. Objectives of the study

1.8.1. General objective

To investigate taxonomic relationships, determination of antimicrobial activity and identification of bioactive compounds in ten indigenous Kenyan *Plectranthus* species in the *Coleus* clade.

1.8.2. Specific objectives

1. To investigate leaf micromorphology (stomatal distribution and leaf anatomy) of ten indigenous Kenyan *Plectranthus* species in the *Coleus* clade.
2. To carry out a molecular phylogeny of ten indigenous Kenyan *Plectranthus* species in the *Coleus* clade using *Rbcl* and *MatK* DNA base sequences.
3. To evaluate antibacterial and antifungal activity of ten indigenous Kenyan *Pectranthus* species in the *Coleus* clade.
4. To isolate, elucidate and characterize antimicrobial compounds from Kenyan *Plectranthus* species within the *Coleus* clade.

2. CHAPTER TWO: LITERATURE REVIEW

2.1. MOLECULAR PHYLOGENY OF *Plectranthus* SPECIES

2.1.1. Introduction

Molecular phylogeny is a branch of phylogeny (evolutionary relationships between species) that analyses hereditary molecular differences, mainly in DNA sequences although RNA and proteins can also be used to gain information on an organism's evolutionary relationships (Dowell, 2008). Molecular phylogenetics employ molecular and statistical techniques to infer evolutionary relationships among species based on their DNA sequences (Hajibabaei *et al*., 2007). Results of a molecular phylogenetics analysis are expressed by use of phylogenetic trees. Similarity of biological functions and molecular mechanisms in living organisms strongly suggests that species descended from a common ancestor.

Molecular phylogeny uses the structure and function of DNA molecules and how they change over time to infer these phylogenetic relationships (Murphy *et al*., 2001). Molecular phylogeny emerged in the early 20th century but did not take up until the 1960s, with the advent of protein sequencing, DNA amplification and sequencing, electrophoresis in addition to other molecular biology techniques (Avise, 2012). In the last two decades, computers have become more powerful and more generally accessible and researchers have been able to develop sophisticated computer algorithms (Dereeper *et al*., 2008). This has enabled researchers to analyze and tackle the immensely complicated stochastic and probabilistic problems that define evolution at the molecular level more effectively.

2.1.2. Existing molecular phylogeny of *Plectranthus* and related genera

It is worth noting that very few studies on the molecular phylogeny of *Plectranthus* genus have been conducted. One such study was conducted by Paton *et al*., (2004) on the subfamily Ocimeae based on three plastid genes, *trnL* intron, *trnL-trnF* intergene spacer and *rps16* gene. Ocimeae is classified under the Labiatae family and consist of various genera of which *Plectranthus* is included. Results from Paton *et al*., (2004) showed that, the two genera *Pycnostachy*s and *Holostylon* formed a phylogenetic group which was closely related to *Plectranthus* genus. Elsewhere, Wagstaff *et al*. (1998) carried out a molecular phylogenetic study on species within the Labiatae family based on *rblc* and *ndhF* chloroplast DNA sequences. Results of the study supported the existing classification of Labiatae subfamilies: Nepetoideae, Laminioideae, Pogostemonoideae and Scutellarioideae and confirmed that these four sub families were monophyletic in origin. However, monophyly of subfamilies Viticoideae and Chloanthiodeae was not supported by the phylogenetic trees generated from the *Rbcl* and *ndhF* DNA sequences (Wagstaff *et al*., 1998)

Al-Qurainy *et al*., (2014) conducted a phylogenetic study on the medicinal plant *Plectranthus asirensis* J.R.I Wood, a plant endemic to Saudi Arabia using *rps16, rpoB, Rbcl, nrITS* and *rpoC1* genes. He reported that *P.asirensis* was closely related to *P. barbatus* and *Plectranthus hadiensis* (Forssk.) Schweinf. Ex Spreng. and even more closely related to *P. caninus* and *Plectranthus coeruleus* (Gürke ex Engl.) Agnew based on DNA sequences from the *nrITS* and *rps16* genes. The four *Plectranthus* species constituted a monophyletic group. DNA from four species of *Plectranthus* which include: *Plectranthus grandis* (L.H.Cramer) R.H.Willemse*, P.barbatus, Plectranthus neochilus* Schltr.*, P. amboinicus* was studied using RAPD analysis to show interspecific diversity among the four species (Bandeira *et al.,* 2009). All the four species

were shown to be monophyletic in origin with *P.neochilus* being closely related to *P.amboinicus* while *P.grandis* (L.H.Cramer) R.H.Willemse was closely related to *P. barbatus*. Moreover, Amarasinghe *et al*, (2013) while carrying out a study on the development of barcodes for investigating phylogenetic relationships of *P. hadiensis* observed that, *P.hadiensis* was closely related to *P.ambonicus* and *Plectranthus amicorum* S.T.Blake and even more closely related to both *P.caninus* and *P.barbatus*.

2.1.3. Universal molecular markers in plants

In plants, the mitochondrial genome generally evolves at the slowest rate, the chloroplast genome evolves at a slightly faster rate and the nuclear genome evolves at the fastest rate (Wolfe *et al.*, 1987). There is, of course, substantial rate of variation within genomes, and coding regions evolve more slowly than non-coding regions which include the introns and intergenic spacers apparently due to selective constraints (Kelchner, 2000). As a result, molecular markers such as chloroplast DNA (cpDNA) and genes like *Rbcl, atpB, MatK ndh F* have been used extensively at the family rank and above. (Soltis *et al*, 1999). Non-coding sequences such as introns (*rpL16*, *rpoC1, rpS16* and *trnL, trnK)* and intergenic spacers (*trnT–trnL, trnL–trnF, atpB– Rbcl, psbA–trn H*) are used more often at the lower taxonomic ranks (Nybom, 2004). Given the relatively slow rate of cpDNA evolution, however, even non-coding cpDNA regions often fail to provide significant phylogenetic information at lower taxonomic levels (Shaw *et al*., 2007). *MatK* gene is one of the best DNA barcode regions for species identification (De mattia *et al.,* 2011). It was able to distinguish species of several genera within the family Labiatae (De mattia *et al.,* 2011).

The development and utilization of molecular markers for the exploitation and identification of plant genetic diversity is one of the most key developments in the field of molecular genetic studies. Some of the techniques used to estimate genetic diversity include:

Random Amplified Polymorphic DNA (RAPD), DNA amplification fingerprinting (DAF), Arbitrarily Primed Polymerase Chain Reaction (APPCR), Inter-simple sequence repeat (ISSR) and Amplified Fragment Length Polymorphism (AFLP), Cleaved amplified polymorphic sequence (CAPS) and Single Nucleotide Polymorphisms (SNPs) besides others. These techniques differ specifically in the DNA amplification procedures (Semagn *et al*., 2006)

2.1.4. *MatK* and *Rbcl* molecular markers

Chloroplast genes evolve at a slower rate compared to nucleus genes and are considered as the best genes for systematic studies. Maturase K (*MatK*) and ribulose-bisphosphate carboxylase gene (*Rbcl*) are examples of chloroplast genes which are considered as universal molecular markers and have been used widely in taxonomic studies of angiosperms. Despite both being chloroplast genes assumed to have slow rate of evolution, *Rbcl* gene has a fairly conservative rate of evolution compared to *MatK* which is thought to evolve at a faster rate (Vijayan and Tsou, 2010). Maturase K gene is responsible for the production of Maturase K enzyme which is involved in RNA editing where it catalyzes intron removal from premature RNAs (Wicke *et al*., 2011) while the *Rbcl* gene is responsible for the production of *Rbcl* enzyme (RUBISCO enzyme or RuMPCase) which is produced in plenty in the leaves of plants; RUBICO plays an important role in the first step of carbon fixation (Wicke *et al*., 2011). The full length of either *MatK* or *Rbcl* gene is about 1500 base pairs (bp), however, this full length is not expressed by all angiosperms and majority of angiosperms have these genes extending not more than 1000bp (Barthet and Hilu, 2007; Kress *et al*., 2005).

2.1.5. Importance of molecular characters in phylogenetic studies

The use of molecular characters in phylogenetic studies has several advantages compared to use of morphological, anatomical and biochemical characters. To begin with, DNA and protein sequences are strictly heritable units hence it is easy to determine whether two plant species are related or not. Secondly, molecular phylogeny allows unambiguous description of molecular characters and character states (Hills, 1987). There is also amenability to mathematical modelling and quantitative analysis with molecular characters which may be hard to achieve with biochemical and morphological characters (Huelsenbeck *et al*., 1996). Molecular characters are not influenced by the environment like morphological characters. With molecular characters, homology assessment is easy and one can easily determine monophyletic groups from the phylogenetic trees produced by the characters. Moreover, distant evolutionary relationships can be revealed by use of molecular characters (Hillis and Huelsenbeck, 1992). Lastly, huge amount of molecular data is available and more can easily be obtained for investigating phylogenetic relationships among species. The results of molecular character analysis are simple, can be obtained fast and are very objective compared to those of other markers such as morphological characters which require taxonomic experts to acquire and can be subjective (Ajawatanawong, 2016).

2.1.6. Steps in molecular phylogeny

The following steps are generally followed in molecular phylogenetic studies (Patwardhan *et al*., 2014).

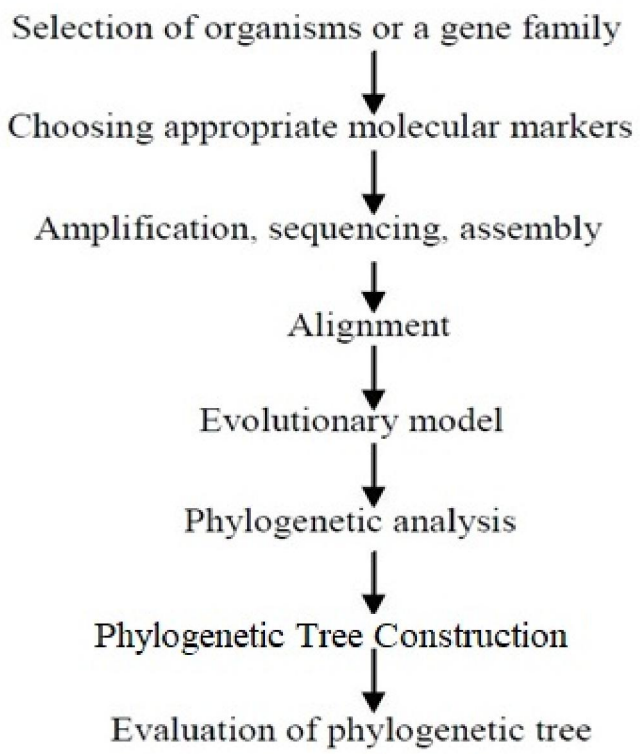


Figure 2.1: Steps in molecular phylogenetic analysis

(Patwardhan *et al*., 2014).

Currently, mounds of genomic data become readily available and consequently molecular phylogenetics is continuing to grow and find new applications (Dowell, 2008). The primary objective of molecular phylogenetic studies is to recover the order of evolutionary events and represent them in evolutionary trees that graphically depict relationships among species or genes over time. *Plectranthus* species are difficult to distinguish morphologically and many species have been wrongly identified and given different names. Use of morphological characters is not

sufficient enough to delimit the genus successfully. Molecular and biochemical markers are better in studying interspecific variation compared to anatomical or morphological markers. So far no study on molecular phylogeny of the Kenyan *Plectranthus* species has been conducted. By comparing the same gene sequence across species within a genus, a phylogenetic tree can be constructed which can support or give new insights into the existing classification. As a result, the current study used molecular characters to classify ten species in the *Coleus* clade within the *Plectranthus* genus. The molecular characters used in the study included DNA sequences of two chloroplast genes: *MatK* and *Rbcl* genes.

2.2. LEAF MORPHOLOGY/MICROMORPHOLOGY OF *Plectranthus* SPECIES

2.2.1. Morphological characters and phylogeny

Morphological phylogenetics involve use of morphological features of organisms in the construction of phylogenies of such organisms. Morphological phylogenetics is important because it helps in the collection of morphological data which can help in resolving phylogenetic relationships of fossil taxa in the future (Jenner, 2004). Most species have evolved and others are now extinct. In addition, most of the extinct groups were diverse and different from their closest existing relatives. Although molecular and chemical data has proved vital in most phylogenetic studies of plant and animal species, the phylogenies of most fossil taxa will in most cases be determined by phylogenetic studies of their morphological data (Novacek and Wheeler, 1992). Another compelling reason why morphological data is still used in phylogenetic studies is the fact that extant species that are still difficult to include in molecular studies. For example, some species once collected need to be preserved and this makes obtaining DNA for molecular work difficult. Other plant species may not be collected again because of limited distribution, their habitat maybe destroyed or because they may become extinct.

As a result, with the current technology, morphological analysis of species with respect to phylogeny remains the only way one can know anything about the relationships of many species (Donoghue and Alverson, 2000). Moreover, technological barriers which limit researchers from obtaining significant amount of DNA for subsequent amplification show that morphological characters are important in phylogenetic studies. Till a point is reached when molecular phylogenetic relationships are recreated without errors, it is still vital to conduct laborious, morphology based phylogenies (Hillis and Wiens, 2000; Jenner, 2004). Morphological data is also important for comparison with molecular or biochemical data to establish whether the phylogenies constructed from both data are similar.

2.2.2. Diagnostic morphological/micromorphological characters in plants

In plants, there are many varied morphological characters such as leaf and floral characters which can be used in describing species and identifying them. However, within the lower taxonomic ranks, notably at the genus and subspecies levels, the characters among the different species tend to overlap and it may be difficult to differentiate the taxa from the general characters. Hence, it is important to identify and use only the diagnostic characters for morphological phylogenetic analysis. For example, the epidermis has a number of important diagnostic characters that present important hints for species identification such as size, shape and stomata orientation, guard cell and epidermal cell wall structure as well as trichome type (Loza-Cornejo and Terrazas, 2003).

In genera such as *Stachys*, *Teucrium* and *Chelonopsis* within Labiatae, morphology of trichomes has been used in infrageneric classification (Salmaki *et al*., 2009; Xiang *et al*., 2010; Navarro *et al*., 1999). Indumentum characteristics, composition, density and distribution of trichomes, qualitative and quantitative characteristics of the stomata are likewise informative in

interspecific phylogenetic studies (Zoric *et al*., 2009). Cuticle ornamentation and epicuticular wax composition are other important distinguishing taxonomic characters. Other leaf anatomical characters which are of high diagnostic value include characters such as midrib position, nature of the spongy mesophyll. Floral characters and pollen morphology are diagnostic as well. Pollen morphology likewise provides a number of diagnostic features which can aid in phylogenetic studies and identification of species (Duarte and Lopes, 2007).

2.2.3. Existing micromorphological studies of *Plectranthus* and related genera

Several micromorphological studies have been conducted on *Plectranthus* and related genera. To begin with, comprehensive morphological studies of trichomes have been done on species of Labiatae family, notably the genus *Salvia* (Bisio *et al*., 1999; Corsi and Bottega, 1999). For example, ordinary caulinar secondary growth has been observed in many dicotyledons and has been reported in *Plectranthus neochilus* (Evert, 2006). Rectangular transection is also frequently observed in members of Labiate and also presence of collenchyma in the four angles has also been observed in the family as well (Cronquist, 1981). A quadrangular cross-section of the stem has been observed in *P.barbatus* and *P.neochilus* (Duarte and Lopes, 2007). In *P. barbatus* stem, the endodermis has been reported to contain many amyloplasts and the vascular bundle arrangement of *P. neochilus* has a striking similarity with that of *P. barbatus* as reported by Duarte and Lopes (2007). Such similarity has also been observed by Evert, (2006) whereby the vascular bundles in *Coleus* are primarily formed in the fascicular regions.

With regard to leaf anatomy, members within the Labiatae family have diacytic and anomocytic stomata both on the abaxial and adaxial surfaces although tetracytic stomata have been observed in the family as well (Duarte and Lopes, 2007). *P. neochilus* has been reported to have diacytic stomata (stomata surrounded by two cells) on both surfaces while *P. barbatus* has been

reported to have anomocytic stomata where stomata are surrounded by many subsidiary cells (Duarte and Lopes, 2007). In *Plectranthus australis,* diallelocytic stomata have been reported where three or more cells of different sizes at the right angles of the guard cells are found surrounding the stomata (Metcalfe and Chalk, 1988).

Micromorphological studies of trichomes of Labiatae revealed that different types of glandular and non-glandular trichomes occur in the family (Fritsch *et al*., 1950). Glandular trichomes are responsible for the production of essential oils, flavone aglycones and resiniferous acids which protect the plant from herbivores, pathogens as well as attracting insects for pollination. For example, peltate trichomes which have uniform morphology and both capitate glandular and non-glandular trichomes have been reported in the family by Werker, (1993). Capitate trichomes have also been reported in *Plectranthus madagascariensis* (Pers.) Benth., *P. ornatus* and in *P. barbatus* (Duarte and Lopes, 2007). Peltate trichomes of *P. ornatus* have been reported to be found only on the abaxial leaf surface while in *P. madagascariensis* they occur on both sides of the leaf (Ascensão *et al*., 1999).

With regard to organization of the leaf chlorenchyma, isobilateral, centric or dorsiventral mesophyll has occasionally been reported in Labiatae family (Fritsch *et al*., 1950). Dorsiventral mesophyll has been reported in *P. barbatus* while homogenous mesophyll occurs in *P. neochilus* (Duarte and Lopes, 2007). In homogenous mesophyll, the leaf anatomy layers are very hard to differentiate and this kind of mesophyll is predominant in those species with succulent leaves (Khalik and Karakish, 2016). Epidermal cell shapes in *Plectranthus* can either be polygonal, where they resemble polygons or sinuous, where they have many curved edges while midrib may be convex, concave, plain convex, biconvex or concave-convex (Duarte and Lopes, 2007). Labiatae is a very large family of angiosperms which is cosmopolitan and member species are found in

nearly all habitats and altitudes. *Plectranthus* is one of the largest genera of Labiatae in the Nepetoideae subfamily under the tribe Ocimeae. The estimated total species within the *Plectranthus* genus are about 300 species distributed in the tropical and sub-tropical regions of the world (Rice *et al.*, 2011).

2.3.4. Description of the ten *Plectranthus* species

Majority of species within *Plectranthus* genus have succulent leaves and stems and are aromatic due to grandular trichomes present on their leaves. Stems are quadrangular, leaves are simple, broad, oval-shaped with a tapering tip and very thick; they are thickly studded with hairs and the lower surface usually has the most numerous glandular hairs. Flowers are borne on a short stem, pale purplish in dense whorls at distant intervals in a long slender raceme (Paton *et al*., 2009). Some diagnostic morphological characters described by Paton *et al*. (2009) for identifying the ten species under investigation include: presence of succulent leaves and stems, height of the plant, number of flowers in the inflorescence, number of cymes, pedicel, calyx, corolla, petiole length, leaf apex type, bracts, nutlet color, nature of staminal filaments and leaf blade as well as the presence of egrandular hairs. Variations of these morphological characters in the ten *Plectranthus* species have been summarized in Table 1 overleaf.

Table 2.1: Description of the ten *Plectranthus* species

Species	Succulent leaf	Height(m)	No. of inflorescence Flowers	Cymes	Pedicels length (mm)	Calyx length (mm)	Corolla length (mm)	Petiole length (mm)	Leaf apex	bracts	Nutlet color	Staminal filaments	Leaf blade	Succulent stem	Egrandular hairs
P.babatus	+	1-5	10-14	5-7	3-7	3-5	8-26	3-50	Ob/rd	ov/api	Brown/black	fused	El/ov	+	An/pt
P.caninus	+	0.1-0.6	6	3	2	3-5	6-14	3-10	Ob/rd	ov	Grey/black	Fused	El/ov	-	Rt/pt
P.montanous	+	0.4-2.5	10-40	5-20	2-3	1.5	3-5	0	Ob/rd	El/ov	Brown/red	free	El/obv	+	Rt/pt
P.otosegioides	+	0.3-0.5	6	3	6-7	5	11-15	3	Ob/rd	Obv/El	Brown/red	fused	Ov/El/Obv	_	pt
P.lanuginousus	+	0.3-1	6-12	3-6	3-6	3-4	8-16	5-30	Ob	EL	Brown/black	fused	Triangular	_	pt
P.pseudomarrubioides	+	1	10-14	5-7	1-3	2	5-9	1-15	Ob	El	Brown/red	free	Rhombic	+	Rt/pt
P.edulis	-	0.3-1.2	6-10	3-5	3-5	2-3	6-8	1-20	Ob/rd	ov	Brown/black	fused	Ov	_	Unknown
P.ornatus	+	0.4-1.5	6	3	3	5-6	14-22	2-10	Ob/rd	ov	Grey/black	fused	El/ov	_	An/pt
P.aegyptiacus	+	0.5-2.5	6-24	3-12	3-10	2-3	8-15	5-20	Ac/rd	ov	Brown/red	fused	Triangular	_	An/pt
P.amboinicus	+	0.3-0.5	10-20	5-10	-3	3	4-15	2-13	Ob/rd	Ov/api	Brown/red	fused	Ov	+	rt/pt

Morphological characters obtained from latest *Plectranthus* classification in the FTEA by Paton *et al.,* 2009.

Key: + (Present); - (Absent); Ob(Obtuse); rd (round); Ac (acute); Ov (Ovate); api (Apiculate); El (Elliptic); Obv (Obovate); An (Antrose); pt (patent); rt (retrose)

Morphological characters have been widely used by taxonomists to identify species. However in closely related species, these morphological characters may differ by a small margin and it may be difficult to differentiate such species hence vital diagnostic characters are needed to delimit closely related species appropriately. Studies of leaf micromorphology can aid in determination of such diagnostic morphological characters which can aid in plant identification and phylogenetic studies. Moreover, species of *Plectranthus* are difficult to classify due to lack of unique morphological features to differentiate not only among individual species in the genus but also among the closely related species in other genera. This has led to many taxonomic hitches in the specifying species leading to placement of these species in closely related genera. Since there is little information available on leaf micromorphology in *Plectranthus*, this study aimed to investigate differences in leaf micromorphology and stomatal distribution of ten *Plectranthus* species in the *Coleus* clade in order to evaluate the taxonomic importance of these characters in intraspecific delimitation of Kenyan *Plectranthus* species.

2.3. ANTIMICROBIAL ACTIVITY AND PHYTOCONSTITUENTS OF *Plectranthus* SPECIES

2.3.1. Introduction

Plectranthus species are extensively used in various parts of the world as ornamentals, source of food and have numerous medicinal uses (Rice *et al*., 2011). About 85 % of all species of *Plectranthus* known to man have been reported to have medicinal value (Lukhoba *et al.*, 2006). Majority of species within *Plectranthus* are used in traditional medicine by various communities to treat a variety of diseases (Matu and van Staden, 2003) and the potential medicinal and economic uses of *Plectranthus* are of great interest (Gasper-marques *et al.,* 2006). Stems, leaves and tubers of different *Plectranthus* species are used to treat a variety of diseases which are classified into 13 categories as described in Economic Botany Data Collection Standard (Cook, 1995). For instance, *P. barbatus* is one of the most important species in the genus widely cited because of its use in the treatment of many diseases (Rice *et al.*, 2011). Besides their medicinal value most *Plectranthus* species have low toxicity in both man and animals (Rice *et al*., 2011).

Plectranthus genus is widely used in horticulture where more than 57 species have been described (Van Jaarsveld, 2006). The genus has also attracted the interest of researchers in breeding experiments aimed at coming up with novel flowers for the horticultural industry (Rice *et al.*, 2011). For example, *P. oertendahlii* and *P. parvifolius* are two species in the genus of horticultural interest which are frequently grown on hanging baskets (Rice *et al.*, 2011). In addition to being used in ethnomedicine, species like *P.barbatus* are also used in ethnoveterinary medicine in the management of livestock diseases; for instance, *P.barbatus* is used to manage east cost fever by the Maasai people of Kenya (Ole-Miaron, 2003). Various bioactive phytoconstituents, including saponins, monoterpenes, sesquiterpenes, terpenes and phenolic compounds have been isolated from *Plectranthus* species (Abdel-Mogib *et al*., 2002). *Plectranthus* has a wealth of

ethnomedicinal species which forms a basis for natural product research. Furthermore, the wide variety of ailments treated by species within *Plectranthus* is an indication of the medicinal richness of the genus and hence the scope of drug development from this genus is endless (Rice *et al*., 2011).

2.3.2. Ethnopharmacological uses of *Plectranthus* species

Plectranthus species have been used in traditional medicine for the management of various diseases. For example, *P.barbatus* and *P.amboinicus* are used in the management of toothache, stomach ache, vomiting as well as in the management of mouth and throat infections (Kokwaro, 2009). *P. aegyptiacus* is also used to treat stomachache (Pakia and Cooke, 2003). *P. barbatus* and *P. amboinicus* have been used in the treatment of burns, sores, allergies, wounds and insect bites (Harsha *et al*., 2003). *P. barbatus* is reported to treat wounds and ringworms (Chifundera, 2001). *P.barbatus* has been used to manage common cold, respiratory tract complications (Rajendran *et al*., 1999) and malaria (Nguta *et al*., 2010). With respect to respiratory diseases, *Plectranthus amboinicus* has been reported to be frequently used in the management of chronic coughs, sore throat, asthma and bronchitis in the Carribean and India (Lukhoba *et al*., 2006; Hutchings *et al.*, 1996). Similary *P. aegyptiacus* has been used in the treatment of laryngitis and sore throats in Saudi Arabia (Al-Yahya *et al*., 1985).

Elsewhere, Rahman *et al*. (2004) reported that *P. montanus* is used in the treatment of sore throats while a concoction from the roots of *P. caninus* has been used in the management of coughs in Kenya (Githinji and Kokwaro, 1993). *P. barbatus* has been reported to treat syphilis in Central Africa (Cos *et al*., 2002) and similarly *P. amboinicus* is often used in the management of urinary tract infections in India and by the Amazon tribes (Yoganarasimhan, 2000). The Giriama of Kenya use *P. aegypticiacus* to treat sexually transmitted diseases (Pakia and Cooke, 2003). A concoction

prepared from both *P. montanus* mixed with *Microglossa oblongifolia* O. Hoffm. has been used to relieve fever and severe headache as reported by Kokwaro, (2009). *Plectranthus* species have also been employed in the management of eye and ear complications. For example, the leaves of *P. amboinicus* and *P. barbatus* can be rubbed onto eyes to manage conjunctivitis and other forms of eyes inflammation (Chifundera, 2001). Majority of *Plectranthus* species have medicinal properties and in particular *P.barbatus* and *P. amboinicus* have been reported to have the widest ethnomedicinal uses (Lukhoba *et al*., 2006).

2.3.3. Phytochemical composition and bioactivity of *Plectranthus* species

Many species of *Plectranthus* have been reported to have various bioactivities. *Plectranthus* has valuable bioactive compounds. For instance, the aromatic nature of the genus is attributed to essential oil production. Chemically active constituents in *Plectranthus* species are alkaloids, sesquiterpenes, terpenes and phenolic compounds. Terpenes are the widely studied phytoconstituents of *Plectranthus*. For example, diterpenes isolated from *Plectranthus* species have been reported to have antifungal activity (Simoes *et al.* 2010), insect repellents (Grayer *et al.*, 2010), antiplasmodial activity (Van Zyl *et al.*, 2008) and anticancer activity (Marques *et al*., 2002). A diterpene, 6,7-dehydroroyleanone has been isolated from the essential oils of the leaves and flowers of *P. madagascariensis* and is associated with bactericidal activity against *Bacillus subtilis*, *Staphylococcus aureus, Micrococcus* sp. and *Yersinia enterocolitica* (Ansensao *et al*., 1998).

Plectranthus glandulosus oil and one of its major compounds; fenchone have been investigated for their anti-repellent activity against the larger grain borer; *Prostephanus truncatus* (Horn) and two strains of another grain storage insect pest *Sitophilus zeamais*. The essential oil from the plant was observed to possess a maximum percent repellency of 100% for the three

insects, while fenchone caused 55–60% repellency to *S. zeamais* and 80% repellency to *P. truncates* (Nukenine *et al*., 2010). Bioassay guided fractionation of the ethyl acetate extract of *Plectranthus ecklonii* Benth. led to isolation and identification of two abietane ditepenes; parvifloron D and parvifloron F with minimum inhibitory concentrations (MIC) values of 15.6 and 31.2 µg/ml respectively against *Listeria monocytogenes.* The same compounds had 190 and 95 µg/ml MIC values against *Mycobacterium tuberculosis* (Nyila *et al*., 2009). The ethyl acetate extract of *P. ecklonii* and its isolated compounds were also tested for their activity on tyrosinase inhibition and the two compounds were also found to inhibit the activity of tyrosinase (Nyila *et al*., 2009).

In another study, essential oil of *P. amboinicus* has been reported to have a promising antimicrobial and anti-inflammatory activity. Gas chromatography-mass spectroscopy (GC-MS) analysis of the essential oil of *P.amboinicus* identified the major compounds present as Carvocrol, Thymol, Cis-Caryophyllene, t-Caryophyllene and p-cymene (Manjamalai *et al*., 2012). Up to ten (10) natural abietanes isolated from the acetonic extract of *Plectranthus grandidentatus* Gürke*, Plectranthus hereroensis* Engl. and *P. ornatus* were evaluated for antibacterial activity against methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus faecalis* (VREF) by Gasper-Marques *et al*. (2006). The results showed that coleon U, horminone, 7*α*-acetoxy-6*β*-hydroxyroyleanone had the highest antibacterial activity with MIC values ranging from 0.98 to 15.63 µg/ml and 15.63 to 31.25 µg/ml for MRSA and VREF clinical strains respectively (Gaspar-Marques *et al*., 2006). Mota *et al*. (2014) investigated the bioactivity and essential oil composition from *P. barbatus, P. ornatus* and *P. neochilus* and reported that monoterpene hydrocarbons and sesquiterpene hydrocarbons were the main fractions of the essential oil in all three species. The major components in *P. barbatus* essential oil were *α*-Pinene,

oct-1-en-3-ol, *β*-pinene and *β*-caryophyllene while *α*-terpenyl acetate, *α*-thujone, *β*-caryophyllene, *β*-pinene and *α*-pinene were the major component in *P. neochilus* while in *P.ornatus* essential oils, oct-1-en-3-ol, *β*-pinene, *α*-pinene and *β*-caryophyllene were the major components. The results showed that the essential oils from the three species had antioxidant and antimicrobial activities against *Gram*-positive bacteria (Mota *et al*., 2014).

Nineteen components have been identified from the essential oil of *P.barbatus* by Govindarajan *et al*. (2016). Eugenol, α-pinene and β-caryophyllene were found to be the major compounds in the essential oil of *P. barbatus*. The three major essential oils components were found to have a significant toxic effect against larvae of *Anopheles subpictus*, *Aedes albopictus* and *Culex tritaeniorhynchus* (Govindarajan *et al*., 2016). Volatile oils, terpenoids especially diterpenes and polyphenolic compounds such as flavonoids and phenolic acids have been isolated from different *Plectranthus* species (El-hawary *et al*., 2012). *P. amboinicus* has also been subjected to a phytochemical analysis which led to isolation and identification of several flavonoids such as 3-methoxy genkwanin, *p*-coumaric acid, crisimaritin, caffeic acid, apigenin, taxifolin, rosmarinic acid and 5-*O*-methyl-luteolin (El-hawary *et al*., 2012). From the study, *P. amboinicus* was reported to possess significant antioxidant, analgesic, diuretic, anti-inflammatory, cytotoxic and antimicrobial activities (El-hawary *et al*., 2012). Further studies on *Plectranthus strigosus* Benth led to the isolation and identification of terpenes and phenol esters such as *ent*-16-Kauren-19-ol , xylopic acid, xylopinic acid , hinokiol , 4β,6β-dihydroxy-1α,5β(H)-guai-9-ene, 4β,6β-dihydroxy-1α,5β(H)-guai-10(14)-ene and *ent*-16-kauren-19-oic acid. A bioactivity study reported that *ent*-16-Kauren-19-ol and *ent*-16-kauren-19-oic acid from *P. strigosus* have herpetic inhibitory properties (Gaspar-Marques *et al*., 2008).

Members of *Plectranthus* genus have been used widely in traditional medicine in the treatment of various diseases (Abdel-Mogib *et al*., 2002). Various researchers have identified phytoconstituents in various species of *Plectranthus* with various bioactivities. Although, most members within the *Coleus* clade have ethnomedicinal importance; majority of the species have not been investigated for their phytochemical composition and bioactivity. Closely related species may possess similar phytoconstituents and consequently may display similar bioactivities (Si *et al*., 2006). As a result, the current study sought to isolate, elucidate and characterize antimicrobial compounds through bioassay guided fractionation of *Plectranthus* species crude extracts.

CHAPTER THREE: MATERIALS AND METHODS

3.1. Collection of *Plectranthus* species samples

Leaves of ten *Plectranthus* species grouped under the *Coleus* clade were collected from various geographical regions of Kenya. *Coleus* clade of *Plectranthus* genus was selected because it is faced with most taxonomic difficulties. Following the information available in the flora of tropical East Africa on *Plectranthus* genus and also from already available nomenclatural types in the East African herbarium, it was possible to identify the specific locality of the species and collect them. Collection was done in 2014 from the following geographical regions/FTEA floral regions (Figure 2.1): *Plectranthus barbatus* (Nairobi/K4), *Plectranthus edulis* (Vatke) Agnew (Trans Nzoia/K3), *Plectranthus ornatus* (Trans Nzoia/K3), *Plectranthus caninus* (Nairobi/K4), *Plectranthus pseudomarrubioides* (Naivasha/K6), *Plectranthus otostegioides* (Olorgesailie/K6), *Plectranthus amboinicus* (Nairobi/K4), *Plectranthus aegyptiacus* (Muranga/K4), *Plectranthus montanous* (Makueni/K4), *Plectranthus lanuginosus* (Nairobi-Ngong/K4).

Collected species were carried in polythene bags from the site of collection to University of Nairobi where they were shade dried. Collection and identification of the species were done with the help of Mr. Patrick Mutiso, a taxonomist from the University of Nairobi and Mr. Mwadime Nyage, a taxonomist from the National Museums of Kenya.Voucher specimens were deposited in Nairobi University Herbarium (NAI). The following are the voucher specimen numbers of the specimens collected: *P. barbatus* (FM2014/01), *P .edulis* (FM2014/02), *P. ornatus* (FM2014/03*), P. caninus* (FM2014/04), *P. pseudomarrubioides* (FM2014/05), *P. otostegioides* (FM2014/06), *P. amboinicus* (FM201407), *P. aegyptiacus* (FM2014/08), *P. montanous* (FM2014/09), *P. lanuginosus* (FM2014/10).

Below is a map showing the various regions (K1 to K7) of the Flora of Tropical East Africa where the species were collected.

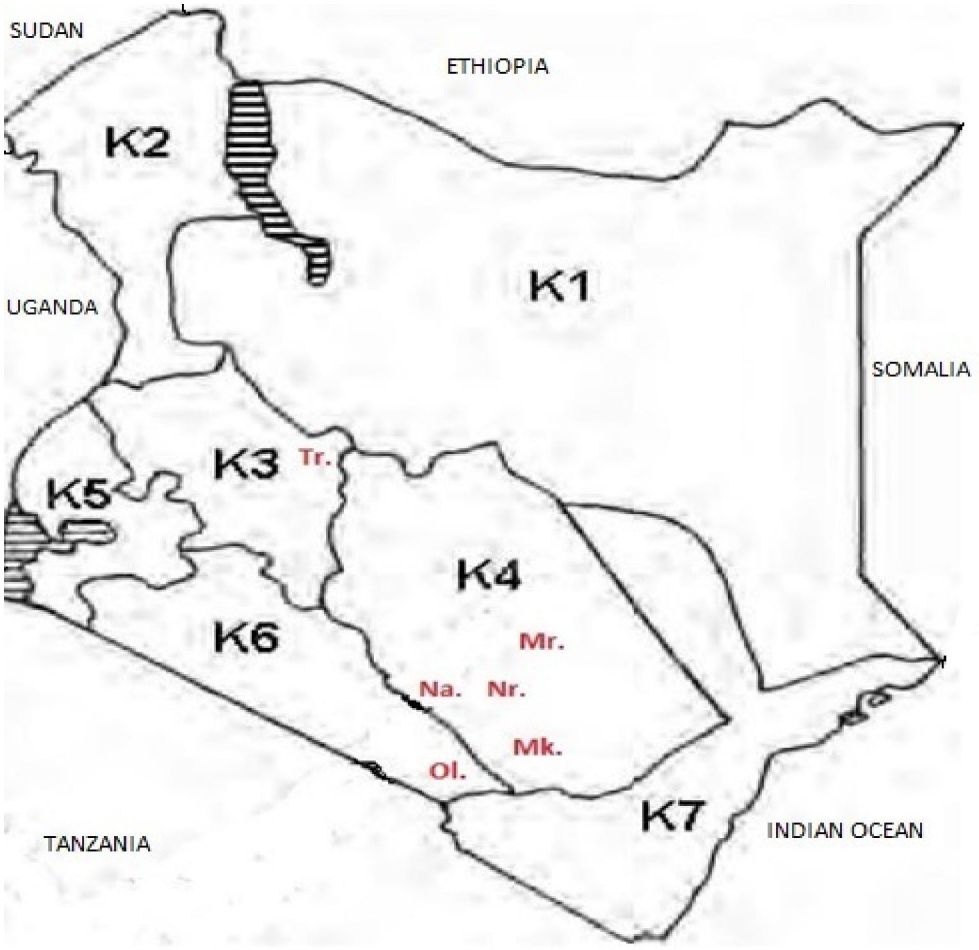


Figure 3.1: Specific localities where *Plectranthus* species were collected within the regions of the Flora of Tropical East Africa

Tr. : Transnzoia/K3 (*Plectranthus edulis, Plectranthus ornatus*), Na.: Naivasha/K4 (*Plectranthus pseudomarrubioides*), **Nr**.:Nairobi/K4 (*Plectranthus lanuginosus, Plectranthus caninus, Plectranthus amboinicus, Plectranthus barbatus*), Mr.: Muranga/K4 (*Plectranthus aegyptiacus*), Mk.:Makueni/K4(*Plectranthus montanous*), Ol.: Olorgesailie/K6(*Plectranthus otostegioides*).

3.2. Deoxyribonucleic acid (DNA) extraction

Leaves meant for DNA extraction were washed with tap water to remove any debris on their surfaces. DNA was extracted from fresh leaves of the collected *Plectranthus* species using the Cetyltrimethyl Ammonium Bromide (CTAB) DNA extraction method as follows: 200 mg of leaves from each species were ground to a fine paste in 500µl of CTAB buffer. The CTAB/plant extract mixture was transferred into a microfuge tube and incubated for 1 hour at 65 °C in a water bath. This was followed by spinning the mixture at 12054g for 10 minutes to spin down cell debris after which the supernatant was transferred to clean microfuge tubes. In each tube, 500µl of Chloroform was added and the solution was gently mixed by inversion. After mixing, the tubes were spun at 12054g for 8 minutes. The upper aqueous phase was then transferred to clean microfuge tubes. In each tube, 50 µl of 7.5 M Ammonium Acetate was added followed by 500 µl of ice cold absolute ethanol. Gentle inversion of the tubes several times was done to precipitate the DNA. The tubes were then stored for 1 hour at -20°C to enhance the DNA precipitation.

The tubes were then centrifuged for 8 minutes at 12054g after which the supernatant was discarded leaving behind the pellets. 500 µl 70% ethanol was then added to the tubes containing pellets and then inverted 5-10 times. This was followed by further centrifugation for 2 minute at 12054g after which the supernatant was discarded. The same procedure was followed again but now with addition of 500 µl 95% ethanol and centrifuged for 2 minutes at 12054g. Lastly the tubes containing the DNA pellets were inverted on a clean wipe and allowed to dry for 10-15 minutes, then dried upright but covered by a wipe for 30-45 minutes. The DNA pellets were then hydrated with 50 uL of Tris-HCL + EDTA (TE) buffer and allowed to suspend again overnight at room temperature. After resuspension, the genomic DNA was incubated at 65 °C for 20 minutes to destroy any DNases that may be present and then stored at 4 °C until use.

3.3. Polymerase Chain Reaction (PCR) of two genes in *Plectranthus*

Two chloroplast genes regarded as universal molecular markers suitable for phylogenetic studies for lower taxonomic ranks in plants were targeted. These genes were: Maturase K gene (*MatK*) and large subunit of the ribulose-bisphosphate carboxylase gene (*Rbcl*). Amplification of DNA was carried out using primers of the two genes. To minimize chances of primers failing to amplify the two genes, they were selected from previous phylogenetic studies of species within Labiatae family rather than designing new ones. The primers were *MatK*-390F and *MatK*-1326R for amplifying the *MatK* gene and *Rbcl*-1F and *Rbcl*724R for amplifying the *Rbcl* gene. Primers used to amplify the two genes are tabulated below.

Table 3.1: Primers used in DNA amplification

Targeted gene	Primer name	Primer direction	Primer sequence	Reference
MatK	*MatK*-390F	Forward sequence(5′→3′)	CGATCTATTCATTCAATATTTC	De Mattia *et al*. (2011)
	MatK-1326R	Reverse sequence (3′→5′)	TCTAGCACACGAAAGTCGAAGT	Amarasinghe *et al.* (2013)
Rbcl	*Rbcl*-1F	Forward sequence (5′→3′)	ATGTCACCACAAACAGAAAC	Bruni *et al*.(2015)
	Rbcl-724R	Reverse sequence (3′→5′)	TCGCATGTACCTGCAGTAGC	Li *et al.* (2011)

The primers and synthetic oligonucleotides for DNA amplification were ordered from Inqaba Biotec East Africa (IBEA). A 10 ul master mix reaction volume for PCR was made with addition of 8ul of milli Q water, 0.5 ul of 5pmole/ul primer and 1ul of 5ng/ul of genomic DNA. This was prepared for each of the 10 *Plectranthus* species and placed in the Thermocycler. Once everything was set, the thermocycler was programmed. First denaturation was set for 2 minutes at 94^0C, this was followed by second denaturation for 1 minute at 94^0C. Annealing was set for 45 seconds at a temperature of 57^0C. DNA extension was set for 30 seconds at 72^0C while final extension was set

for 7 minutes at 72 ^{0}C. Lastly, the number of Polymerase Chain Reaction cycles were set to 40. After the amplification cycles were over, a gel electrophoresis was done to confirm whether amplification had occurred after which the PCR products were ready for purification and sequencing.

3.4. DNA Sequencing of amplified genes in *Plectranthus*

PCR product purification and sequencing was performed by Inqaba Biotec East Africa (IBEA). The amplification primers were likewise used as sequencing primers. PCR products were purified using Microcon YM 100 filter devices and bidirectionally sequenced using the ABI 377 automated sequencer. The obtained forward and reverse sequences of the two genes from the ten *Plectranthus* species were then exported to MEGA 6 software (Molecular Evolutionary Genetics Analysis) edited and then aligned prior to phylogenetic analysis.

3.5. Phylogenetic analysis techniques

3.5.1. Assembling and aligning datasets

All the *MatK* and *Rbcl* sequences from the 10 *Plectranthus* species were exported and assembled in MEGA 6 and their ends edited manually to remove wrong bases at the ends which might compromise the quality of the sequences, then aligned using Muscle-codon alignment method (Kumwenda *et al*., 2013). This ensures similar bases and gaps in the sequences from the two genes in the 10 species sequenced lie side by side which is a prerequisite for the construction of phylogenetic trees.

3.5.2. Building phylogenetic trees

Rbcl and *MatK* phylogenetic trees were likewise constructed in MEGA 6. Prior to building a phylogenetic tree an evolution model, a statistical method for analyzing the tree and a test of

phylogeny must be selected. The evolution model followed was maximum composite likelihood model (Tamura *et al*., 2011), while the statistical method used was UPGMA (Unweighted Pair Group Method with Arithmetic Mean) developed by Sneath and Sokal (1973) and the test for phylogeny was bootstrap resampling method (Efron and Tibshirani, 1994). The sequences already in MEGA 6 were subjected to these three parameters and phylogenetic trees were constructed. Maximum composite likelihood is frequently preferred because it aids taxonomists to discriminate among many equally likely assignments by using the posterior probabilities for each probable nucleotide or amino acid assignment and generate the most likely (most parsimonious) tree which fits the data (Tamura *et al*., 2011).

UPGMA uses a step by step clustering algorithm to find two Operational Taxonomic Units (OTUs) that are utmost similar (meaning they have the shortest evolutionary distance and are most similar in sequence) and regard them as a single new composite OTU. This process is repeatedly performed till only two OTUs remain which will be found at the end of the phylogenetic tree. Bootstrap method involves resampling of the original DNA sequences with replacement to generate meaningful statistical distributions which are compared to determine a bootstrap consensus tree. Bootstrapping is used to place confidence intervals on phylogenies. The number of boostrap iterations for the sequences was set at 1000. In addition, the sequences were also compared with sequences of other *Plectranthus* already deposited in the NCBI database to show the phylogenetic position of the 10 *Plectranthus* among the wider *coleus* clade or *Plectranthus* genus in general.

3.6. Stomatal distribution and density in *Plectranthus* leaves

One centimeter cubed of leaf tissue was removed from the middle section of the leaf and placed in a glass boiling tube. This was followed by addition of 5 ml of glacial acetic acid and

hydrogen peroxide in equal parts in sufficient quantity to cover the material, and the tubes were then heated to 70 °C. After 2 hours, the epidermal layers were teased apart from each other, washed in water, and stained with Safranin-O and mounted on microscope slides and observed x 40, x 100 and x 400 magnifications (Davis and Barnett, 1997; Al-Saghir *et al*., 2005). Microscope type used was LEICA DM 500 microscope (a binocular compound microscope with a magnification range of x 40 to x 1000) with a computer plugin and a digital camera capable of photogaphing and displaying magnification field on a computer. Stomatal distribution was determined for each surface by randomly counting them in three different magnification fields in the different *Plectranthus* species collected then the average was calculated.

3.7. Leaf anatomy

To study leaf anatomy, portions of leaf lamina were cut into 4-10 mm^2 sections prior to rehydration. Tissues were then rehydrated in a series of 25% alcohol, 10% alcohol, and distilled water followed by staining in saturated aqueous safranin-O. The stained tissues were then dehydrated in an alcohol series, 50% alcohol and 100% Xylene. The dehydrated tissues were then infiltrated in a series of xylene: paraffin oil (50%:50%) and 100% paraffin oil followed by a second series of molten paraplast. The infiltrated tissues were later embedded in paraffin in casting blocks. Paraffin embedded blocks were then sectioned transversely at 2 µm thickness with razor blades on a rotary microtome (Al-Saghir *et al.*, 2006). Slides were later observed and photographed at × 100 and × 400 magnifications to observe differences in the arrangement of various internal leaf layers (epidermal cells, palisade layer and spongy mesophyll) as well as trichomes among the *Plectranthus* species .

3.8. Scoring of charaters.

Before analysis of leaf micromorphoogy data, the leaf micromorphology characters obtained were assigned values. The values represented character states for each character. The number of values assigned depended on the number of charater states available for each character. Eventually a matrix was created which was subjected to cluster analysis.

3.9. Cluster analysis based on leaf characters

Phenetic analysis (cluster and principal components) is commonly used as a tool to better understand the trends of morphological variations, by indicating relationships among taxa in form of dendrograms or cladograms (Moraes *et al.* 2011). Scored characters in a matrix were subjected to hierarchical cluster analysis in SPSS Version 23 to generate a dendrogram showing linkages between the species based on their character values. The statistical method used was average linkage between groups which is also referred to as UPGMA (Yim and Ramdeen, 2015). This is the same statistical method used in the analysis of *MatK* and *Rbcl* sequences. During cluster analysis, SPSS calculates the smallest average distance between all the available group pairs and subsequently combines two closely related groups. Cluster analysis usually groups organisms together based on their overall similarity. This grouping was used to draw inferences about the taxonomic relationships of the species investigated.

3.10. Evaluation of antimicrobial activity of *Plectranthus* species

3.10.1. Extration and preparation of crude extracts of *Plectranthus* species

Leaves from the ten *Plectranhus* species under study were collected from various geographical regions as described in section 3.1 and dried under shade after which they were ground into fine powder using a blender. 500 g of air dried ground material of leaves from each of

the 10 *Plectranthus* species were extracted by cold maceration with 1 litre of Dichloromethane: Methanol (1:1) in 2.5 liter conical flasks well covered with aluminium foil for 1 week at room temperature (Harborne, 2002). Filtrates were concentrated in a rotary evaporator and then left to dry in a fume chamber to ensure the total extract was free from the extraction solvents (Harborne, 2002). The studied species gave varying yield as tabulated in Table 3.2.

Table 3.2: Yields and percentage recovery from DCM: MeOH (1:1) leaf extracts of *Plectranthus* species

Plant species	Yield (g)	Percentage recovery (%)
P. caninus	8.2	1.7
P. aegyptiacus	7.4	1.5
P. edulis	7.0	1.4
P. pseudomarrubioides	10.1	2.0
P. barbatus	9.4	1.9
P. lanuginosus	6.0	1.2
P. otostegioides	5.8	1.2
P. montanous	7.5	1.5
P. ornatus	8.6	1.7
P. amboinicus	6.7	1.4

3.10.2. Determination of antimicrobial activity of the crude extracts.

Nutrient agar and broth were prepared as follows, 6.5 g of nutrient agar and 6.5 g of nutrient broth powder were measured and dissolved in 1 liter of distilled water simultaneously, and stirred and then autoclaved. After sterilization, the nutrient agar was poured into petri-dishes before cooling while nutrient broth was stored at -4 ^{0}C. Microbes used for determination of the antimicrobial activity of crude extracts were: Methicillin Resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* (ATCC25922), *Bacillus cereus* (ATCC11778), *Aspergillus niger* (ATCC16888) and *Candida albicans* (ATCC10231). Microbes were obtained from School of Biological Sciences at the University of Nairobi. The Microbes already stored at -4 ^{0}C were revived

by subculturing them in agar plates and incubating them at 37 ^{0}C them for 24 hours for MRSA, *B.cereus* and *E.coli* and 72 hours for *A.niger* and *C.albicans.*

3.10.3. Preparation of *Plectranthus* test extracts

The three concentrations (200 mg/ml, 100 mg/ml and 50 mg/ml) were prepared from each of the total extracts prepared from the leaves of the ten *Plectranthus* species according to Nguta *et al*. (2016). Two grams (2g) of the crude extract from each species was dissolved in 5 ml distilled water containing 10% DMSO to make a 5 ml solution of 400 mg/ml. 0.5ml of 400 mg/ml contained 1% DMSO and was diluted with distilled water to make a stock solution solution containing 200 mg/ml with less than 1% DMSO. 200 mg/ml stock solution was subsequently serially diluted with distilled water to get 100 mg/ml and 50 mg/ml. DMSO in prepared extracts was maintained below 1% to avoid carry over effects. It was reported by Trivedi *et al* (1990) that a DMSO concentration of less than 1% in bioassay preparations has no apparent effect on microbial growth. Sterilized paper discs (5mm in diameter) were incubated with each of the three concentrations and allowed to dry under lamina flow hood. Ketoconazole (40 mg/ml) and Amoxicillin (50 mg/ml) were used as the positive controls for the antifungal and antibacterial activity respectively while 1% DMSO was used as the negative control.

3.10.4. Disc diffusion technique

Pure culture inoculum of each of the test microbes were prepared by transferring a loopful of the revived microbes into 10 ml of distilled water in a test tube followed by shaking to ensure even mixing. Using a micropipette, 1 ml inoculum of pure culture of each of the five microbes were transferred to nutrient agar on petri dishes aseptically and spread with a L-shaped glass rod. The discs prior incubated with the crude extracts at different concentrations were then placed

evenly on the nutrient agar and the petri dishes were placed in inverted position and then placed in an incubator set at 37 degrees Celsius. For the bacteria, zone of inhibition was determined after 24 hours while for the fungi, zone of inhibition was determined after 72 hours.

3.10.5. Broth dilution technique

Minimum inhibitory concentration (MIC) was determined by broth dilution in sterile standard test tubes. A 24 hour plate culture of the test microbes were adjusted to 0.5 McFarland turbidity standard using a spectrophotometer. This 0.5 absorbance level is assumed to contain 1 to 2 x 10^8 colony forming units (CFU)/mL (Gomes *et al*., 2003). 4 g of the organic crude extracts of each of the *Plectranthus* species was first dissolved in a 10 ml nutrient broth containing 10% DMSO to make 10 ml of 400 mg/ml. 1ml of 400 mg/ml containing 1% DMSO was diluted with nutrient broth to make a stock solution of 200 mg/ml with less than 1% DMSO. Stock solution was serially diluted from 200 mg/ml to 25 mg/ml using micropipettes. This ensured that the percentage of DMSO in the stock solution and all the other prepared concentrations was below 1 % to avoid carry over effects. The dilutions were then inoculated with 500 µl of test microbes (MRSA, *B.cereus, E.coli, C.albicans* and *A.niger*) and incubated at 37^0C for 24 hours for bacteria and 30^0C for 72 hrs for fungi. The lowest concentration with no visible bacterial or fungal growth was regarded as the MIC for the respective microbe.

3.10.6. Bioassay guided fractionation of *P.barbatus* leaf extracts

Plectranthus barbatus was found to exhibit higher growth inhibition zones compared to the other nine species and was subjected to further investigation. This involved partitioning the crude extract of *P.barbatus* in solvents of increasing polarity, bioassay of the partitions and later

subjecting the partition with higher antimicrobial activity to column chromatography to get pure isolates which were then assessed for antimicrobial activity.

3.10.6.1. Partition Liquid Chromatography (PLC) of *P. barbatus* leaf extracts

Leaves of *P. barbatus* were air dried and ground into powder using a blender. 1000 g of the ground material was subjected to extraction by cold marceration using 80% ethanol for 1 week. Ethanol was then vapourized and remaining crude extract was suspended in distilled water and then subjected to successive partitioning with organic solvents of increasing polarity. Solvents used were petroleum ether, n-hexane, dichloromethane, chloroform and lastly ethyl acetate. A separation funnel was used to separate the partitioned crude containing extracts at each stage. This resulted into five partitions; petroleum ether partition, n-hexane partition, DCM partition, chloroform partition and ethyl acetate partition. The partitions were concentrated in a rotary evaporator and then left to dry in a fume chamber to ensure the total extract was free from the extraction solvents (Harborne, 2002). The resulting partitions were subjected to antimicrobial activity through disc diffusion and broth dilution.

3.10.6.2. Liquid column chromatography (LCC) of the DCM partition of *P.barbatus*

From the preliminary antimicrobial screening *P.barbatus* partitions, DCM partition was found to inhibit the growth of the test microbes better compared to petroleum ether, n-hexane, chloroform and ethyl acetate partitions and hence was selected for Liquid Column Chromatography. Approximately two (2) kg of ground *P. barbatus* leaves were extracted with DCM: MeOH (1:1) for 1 week. The filtrate was concentrated in a rotary evaporator to get 152g of the crude DCM: MeOH organic extract. The extract was mixed with silica gel in the ratio 1:1 in a Buckner funnel and partitioned with hexane to remove most of the fatty acids and chlorophyll,

followed by partitioning with DCM to get DCM partition. The DCM partition was concentrated in a rotary evaporator resulting in 45g of DCM extract which was then subjected to liquid column chromatography. The generated 45 g of DCM extract was adsorbed in 45 g of silica gel, and later loaded in a column packed with 180 g of silica gel and conditioned with the first eluting solvent system.

A TLC profile of the DCM partition was done to determine the best solvent systems to use in running the column chromatography. The solvent systems used were: Ethyl acetate/Hexane (2:8) and Methanol/DCM/Hexane (2:3:5). Ethyl acetate/Hexane solvent system was found to be the best for eluting the DCM partition of *P.barbatus* in a column packed with silica gel. Column was eluted with Ethyl acetate/Hexane solvent system in a step gradient manner starting with 5% ethyl acetate in hexane i.e 5% EtoAC: 95% hexane to 100% EtoAC: 0% hexane. 100 mls of fractions were collected and concentrated in a rotary evaporator and the concentrated fractions were stored in vials. Vials were loosely covered with aluminium foils to ensure continued evaporation, while avoiding contamination. The fractions obtained after running the first column were combined based on their TLC profiles. Combined fractions were further subjected to column chromatography using smaller columns for further isolation in an attempt to get pure compounds. EtoAC: Hexane solvent system was used as the eluting solvent and columns were run with this solvent system with increasing polarity from 5% ethyl acetate in hexane i.e 5% EtoAC: 95% hexane to 100% EtoAC: 0% hexane. 40 mls fraction were collected from the column and were concentrated in a rotary evaporator and subjected to TLC analysis in an attempt to identify isolated compounds. Ethylacetate: Hexane fractions which produced single spots after TLC were cleaned by mixing them with 5 ml of methanol then allowed to cool at room temperature in attempt to crystalize the pure compounds**.** Two compounds were isolated i.e Compound A (1.1g) and B

(2.3g). Isolated compounds were later subjected to antimicrobial activity and their structures elucidated using Nuclear Magnetic Resonance Spectroscopy (NMR) and Mass Spectroscopy (MS).

3.10.6.3. Antimicrobial activity of isolated compounds

Minimum inhibitory concentration of the isolated compounds was determined by broth dilution in sterile standard test tubes. A 24 hour plate culture of the test microbes were adjusted to 0.5 McFarland turbidity standard using a spectrophotometer. Test solution was prepared by dissolving one (1) gram of each of the isolated compounds from *P. barbatus* in a 10 ml nutrient broth containing 10% DMSO to make 100 mg/ml. 1ml of 100 mg/ml contained 1% DMSO and was diluted with nutrient broth to make a stock solution containing 50 mg/ml with less than 1% DMSO. The 50 mg/ml was serially diluted to make 40 mg/ml, 30 mg/ml, 20 mg and 10 mg/ml using micropipettes. The dilutions were then inoculated with 500 µl of test microbes (MRSA, *B.cereus, E.coli, C.albicans* and *A.niger*) and incubated at 37^0C for 24 hours for bacteria and 30^0C for 72 hrs for fungi. The lowest concentration with no visible bacterial or fungal growth was assumed to be the MIC value for the respective microbe.

3.11. Nuclear Magnetic Resonance (NMR) and Mass Spectroscopy (MS)

The isolated compounds were subjected to one dimensional (1D) and two dimensional (2D) NMR. 1D NMR included ^{13}C NMR and ^{1}H proton NMR (PMR) while 2D NMR experiments included COSY (homonuclear correlation spectroscopy), HSQC (Heteronuclear Single Quantum Correlation) and HMBC (Heteronuclear Multiple Bond Correlation) NMR. COSY NMR shows coupling of H protons across single bonds in a compound while HSQC NMR shows coupling between C and H atoms across single bonds in a compound. HMBC NMR was also done to show

the coupling of C and H atoms across multiple bonds in the pure compounds. In addition, high resolution Mass Spectroscopy (MS) was performed to determine the molecular ion peak (M+) equivalent to the molecular mass of the unknown compound (Silverstein *et al*., 2014). Structures of the pure compounds were illustrated in ChemDraw Ultra 8.0 after interpreting the NMR and MS Spectra.

3.12. Compilation of data and data analysis

Analysis of data was performed using SPSS. Using the software; descriptive statistics such as means, standard errors of the mean, variance, range, confidence interval of the mean of inhibition zones were computed and tabulated. In addition, one way ANOVA was performed to determine whether there were significance differences in terms of antimicrobial activity among the *Plectranthus* species/partitions/isolates investigated. Further ANOVA (post hoc ANOVA) was also performed using Dunnett T test to compare the activity of the crude extracts of *Plectranthus* species/partitions/isolates with the antimicrobial activity of the positive controls to determine whether there was any significant difference. Dunnett T test is recommended when there is a control group in an experiment (George and Marlley, 2016). The level of significance/probability level used in the analysis was ≤ 0.05.

CHAPTER FOUR: RESULTS

4.1. *Plectranthus* species investigated

The following ten *Plectranthus* species in the *Coleus* clade were investigated**:** *P. pseudomarrubioides* Willemse*, Plectranthus caninus* Roth*., Plectranthus lanuginosus* (Hochst. ex Benth.) Agnew. *Plectranthus otostegioides* (Gürke) Ryding*, Plectranthus barbatus* Andrews, *P.montanous* Benth*, Plectranthus ornatus* Codd, *Plectranthus edulis* (Vatke) Agnew*, Plectranthus aegyptiacus* (Forssk) C.Chr and *Plectranthus amboinicus* (Lour.) Spreng.

***Plectranthus pseudomarrubioides* Willemse**

P. pseudomarrubioides is an aromatic perennial succulent herb reaching upto a height of 1m. Stems are rounded-quandrangular, succulent, erect and sometimes prostrate. Leaves are spreading, succulent or deflexed, densely pubescent to tormentose with sessile glands. Inflorescence is lax in fruit and has 10-40 flowered verticils while cymes are sessile and are 5-7 flowered, pedicels are 1-3 mm long while calyx is 2 mm long and is purplish and has yellowish sessile glands. Corolla is pale blue with yellowish sessile glands on lobes and is usually 5-9 mm long.

***Plectranthus caninus* Roth**

Aromatic perennial herb and sometimes annual succulent herb 0.1 to 0.6 m tall whose stems arise from a branched taproot. Stems are sprawing to erect or braching to form clumps and have glandular and eglandular hairs. Leaves are fleshy, petiolate, spreading or ascending and often folded along the midrib upon drying and have red sessile glands. Inflorescence is condensed with 6-flowered verticils and cymes are 3-flowered. Calyx is 3-5 mm long, pubescent and have red or yellowish sessile glands while corolla is blue or purple marked white, 6-14 m long with scattered red glands on lobes.

***Plectranthus lanuginosus* (Hochst. ex Benth.) Agnew.**

Aromatic fleshy perennial herb, 0.3 to 1m tall with single or many stems arising from a branched taproot. Stems are usually prostrate, rounded-quadrangular, often purplish with grandular and egrandular hairs. Leaves are succulent, spreading and petiolate and petiole is usually 10-30 mm long. Infloresence is laxous with 6-10 flowered verticils while cymes are 3-6 flowered and are sessile. Calyx is villose, has red sessile glands and is 3-4 mm long while corolla is blue marked purple or mauve and is 1-13 mm long, its corolla lobes have purplish hairs and red sessile glands.

***Plectranthus otostegioides* (Gürke) Ryding**

Aromatic perennial semi-succulent or soft wooded herb, 0.3 to 0.5 m tall but can scramble up to 1.5 m with several stems emanating from a root stock, softly pubescent with red sessile glands. Leaves are petiolate and often become yellow green and are succulent. Inflorescence is a condensed lax with 6-flowered verticils while cymes are 3 flowered. Pedicels are 6-7 mm long, flattened and winged at the base. Calyx is 5 mm long, pubescent and has red sessile glands while corolla is mauve or white with blue markings, its 11-15 mm long and is pubescent with red sessile glands.

***Plectranthus barbatus* Andrews**

P. barbatus is an aromatic perennial herb reaching up to 4 m in height. Stems are erect, ascending and sometimes creeping at the base and have sessile glands. Leaves are spreading, succulent and petiolate with red sessile glands. Petiole is 3-50 mm long. Inflorescence is laxous with 10-14 flowered verticils while cymes are sessile and are 5-7 flowered. Calyx is 3-5 mm long and is pubescent to villose with red to yellowish sessile glands while corolla is blue or purple and is 7-26 mm long and has scattered red glands on lobes.

***Plectranthus montanous* Benth.**

P. montanous is an aromatic perennial succulent herb with rounded quadrangular succulent stems. Stems are erect or prostrate and sometimes clumb forming and are pinkish to dark red. Leaves are succulent and are spreading or deflexed and their blades are elliptic to obovate. Inflorescence has 10-40 flowered verticils. Cymes are sessile and are 5-20 flowered. Calyx is 1.5 mm long with white hairs and sessile glands while corolla is either blue or violet. *P. montanous* reaches a height ranging from 0.4 to 2.5 m.

***Plectranthus ornatus* Codd**

An aromatic softly wooded perennial succulent herb with a height ranging from 0.4 to 1.5 m tall. Stems are erect or straggling, branching sometimes forming cushions and have scattered red sessile glands. Leaves are succulent, spreading or ascending and petiolate. Inflorescence is a condensed lax with 6-flowered verticils while cymes are 3-flowered. Calyx is 5-6 mm long, is purplish, pubescent with scattered yellow and red sessile glands while corolla is purplish, 14-22 mm long with scattered hairs on lobes.

***Plectranthus edulis* (Vatke) Agnew**

Plectranthus edulis is an aromatic perennial herb 0.2 to 2 m tall with several stems arising from a branching rooting system sometimes bearing tubers which are edible. Stems are quadrangular often creeping at the base, swollen at the nodes, glandular and sometimes with purplish hairs. Leaves are spreading or ascending and have a reduced petiole. Inflorescence is usually lax with 6-10 flowered verticils while cymes are 3-5 flowered. Calyx is 2-3 mm long, its pubescent and has red sessile glands while corolla is blue, marked purple on lobes and its length ranges from 13-18 mm.

***Plectranthus aegyptiacus* (Forssk) C.Chr.**

P. aegyptiacus is soft wooded aromatic perennial herb, 0.5 to 2.5 m tall. Stems are rounded-quadrangular, often succulent and leafless at flowering.Leaves are ascending or spreading and succulent with pale sessile glands. Inflorescence is a terminal lax with 6-24 flowered verticils. Bracts have 3-12 flowered cymes. Pedicels are 5-10 m long while calyx is 2-3 mm long with long and short egrandular hairs and pale sessile glands. Corolla is pale mauve or puple and has pale sessile glands.

***Plectranthus amboinicus* (Lour.) Spreng.**

P. amboinicus is also an aromatic perennial herb, 0.3 to 0.5 m tall. Stems are rounded-quadrangular, sometimes purplish, they are succulent, have grandular and egrandular hairs and have pale sessile glands. Leaves are spreading and succulent. Inflorescence is terminal lax with 10-20 flowered verticils and each bract has 5-10 flowered sessile cymes. Calyx is 3 mm long, pubescent with pale sessile glands. Corolla is pinkish white to pale violet and 4-9 mm long and has pale sessile galands on lobes.

Pictures of the ten *Plectranthus* species under study are represented in Figure 4.1 overleaf.

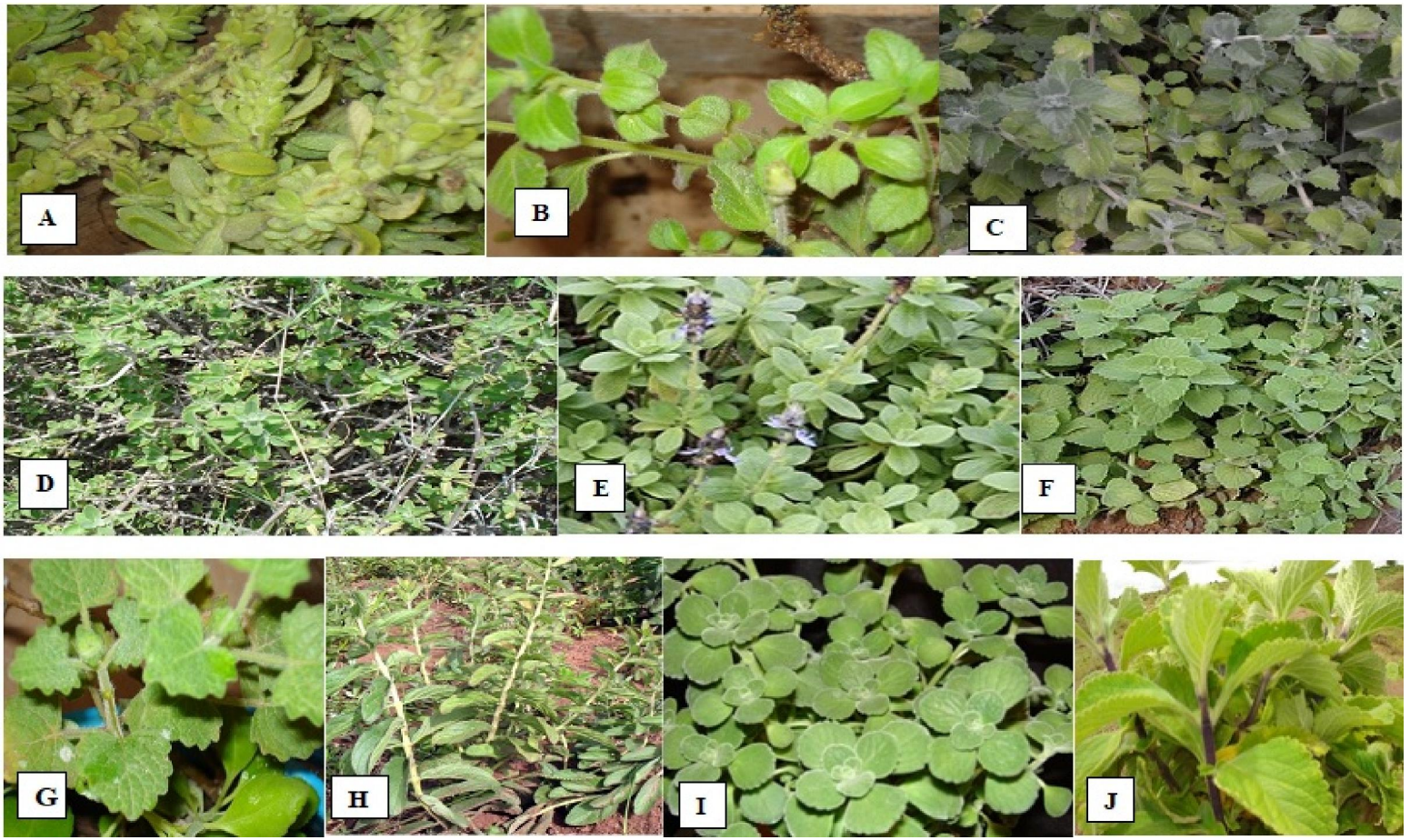


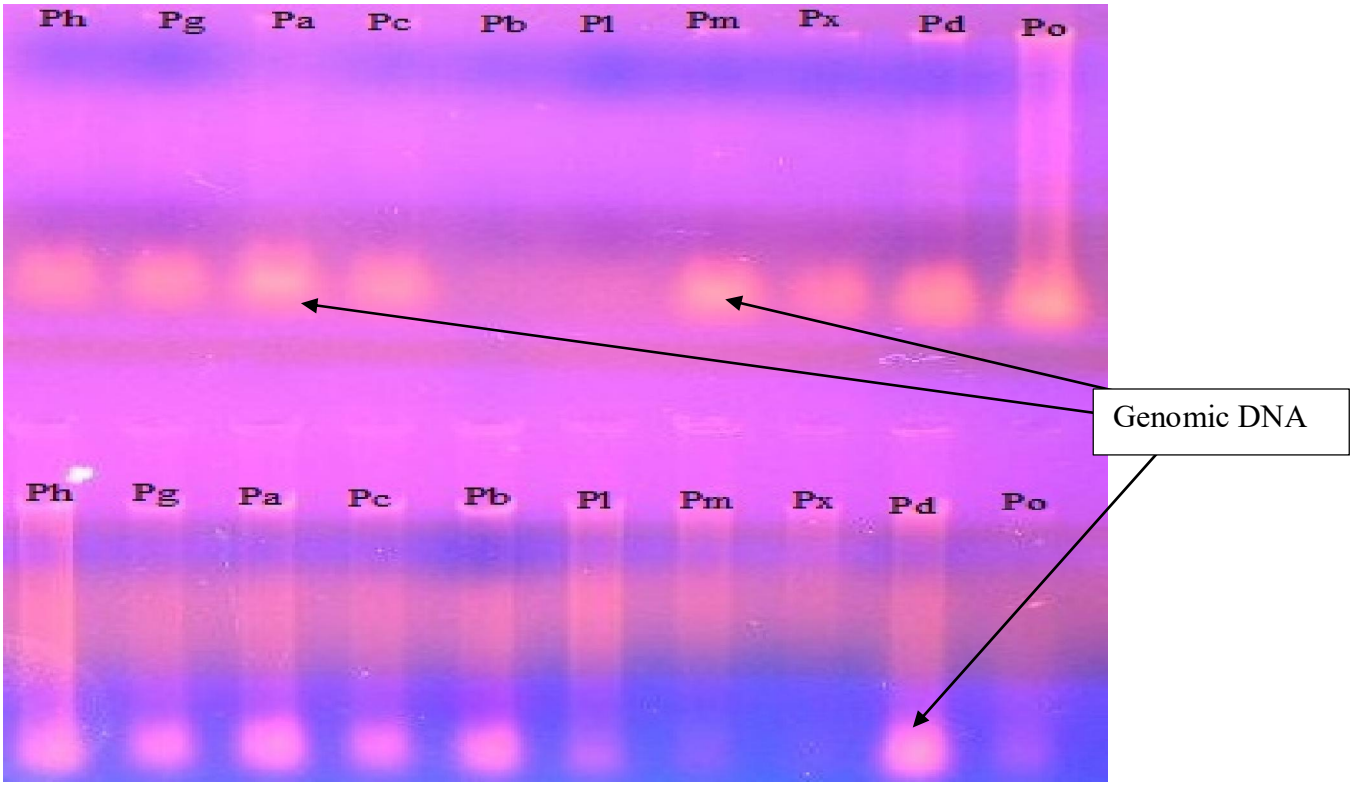
Figure 4.1: Pictures of ten *Plectranthus* species showing aerial parts

A: *Plectranthus montanous*, **B:** *Plectranthus caninus,* **C:** *Plectranthus pseudomarrubioides,* **D:** *Plectranthus otostegioides,* **E:** *Plectranthus ornatus,* **F:** *Plectranthus aegyptiacus,* **G:** *Plectranthus lanuginosus*, **H:** *Plectranthus edulis*, **I:** *Plectranthus amboinicus* **J:** *Plectranthus barbatus*

4.2. Molecular phylogeny of ten *Plectranthus* species

4.2.1. Genomic DNA

Gel electrophoresis was performed on the DNA extract from each of the ten *Plectranthus* species to ascertain the presence or absence of genomic DNA prior to amplification of *MatK* and *Rbcl* genes. A gel electrophoresis image of the Genomic DNA obtained from each of the ten *Plectranthus* species (Figure 4.2). White bands imply presence of genomic DNA implying there was successful extraction of DNA in the ten *Pletranthus* species.



Key: **Ph:** *P. pseudomarrubioides,* **Pg**: *P.ornatus*, **Pa**: *P.edulis,* **Pc:** *P. caninus,* **Pb:** *P.barbatus,* **Pl:** *P. lanuginosus,* **Pm:** *P.montanous,* **Px:** *P.amboinicus,* **Pd:** *P.aegyptiacus,* **P.o:** *P. otostegioides*

Figure 4.2: Gel electrophoresis of genomic DNA from the ten *Plectranthus* species

4.2.2. Sequencing and multiple alignment of *MatK* and *Rbcl* genes

After DNA amplification, sequencing was done at Inqaba Biotech (SA). Results of both the forward and reverse DNA sequences of *MatK* and *Rbcl* genes of the ten *Plectranthus* species were obtained in Applied Biosystems Format (.abi format). Subsequently, they were aligned using MEGA 6 to ensure that similar bases lie side by side among the 10 species. Multiple sequence alignment helped to identify gaps, similar and mismatch regions among the two molecular characters (*MatK* and *Rbcl* genes) which is a prerequisite step in the creation of phylogenetic trees. The following is a portion of multiple alignment of *MatK* gene sequences from the ten *Plectranthus* species in MEGA 6 showing similar and mismatch regions and gaps among the DNA sequences of the ten *Plectranthus* species (Figure 4.3).

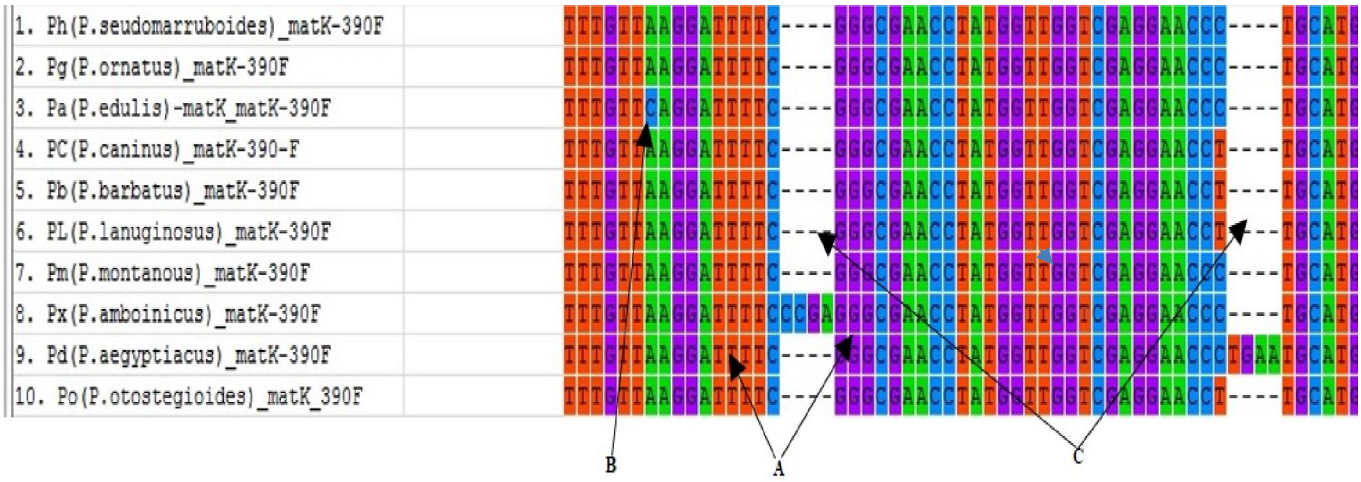


Figure 4.3: Multiple alignment of *MatK* sequences

A: similar regions among the various DNA sequences of *MatK* gene; **B**: a mismatched cytosine base in *P.edulis*; **C**: gaps within *MatK* DNA sequences

Similarly, the following is a portion of multiple alignment of *Rbcl* gene sequences from the ten *Plectranthus* species in MEGA 6 showing similar and mismatched regions and gaps among the ten *Rbcl* DNA sequences (Figure 4.4).

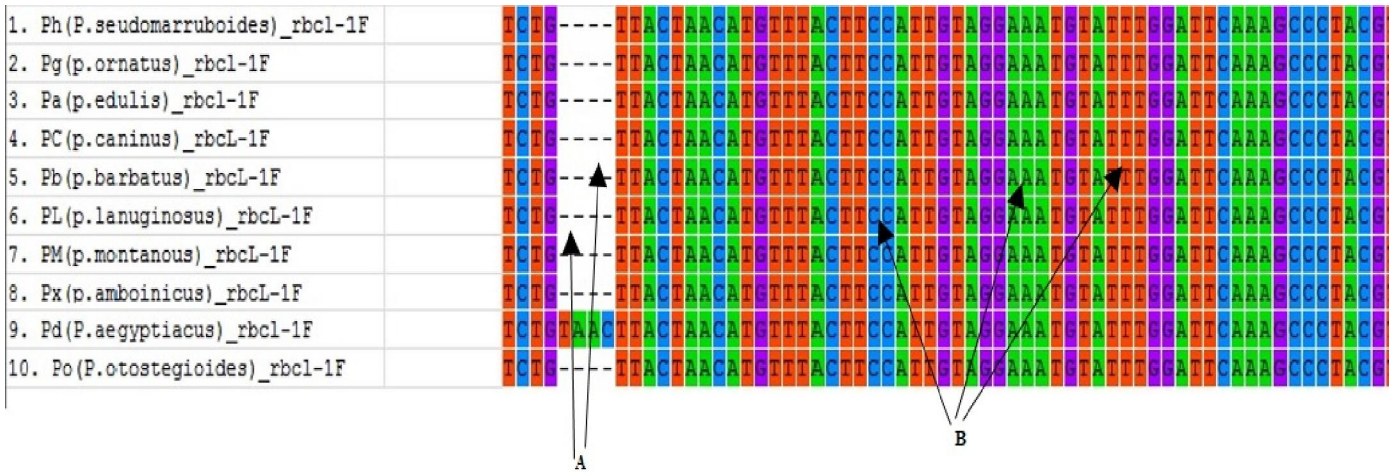


Figure 4.4: Multiple alignment of *Rbcl* sequences

A: gaps with *Rbcl* gene sequences; **B:** similar regions among the various DNA sequences of the *Rbcl* gene

4.2.3. Phylogenetic trees based on *MatK* and *Rbcl* gene sequences

Aligned sequences were used in the creation of the following phylogenetic trees. Overleaf is the most likely phylogenetic tree created based on *MatK* gene sequences from the ten *Plectranthus* species showing various taxonomic relationships (Figure 4.5). The numbers at the nodes are bootstrap values which indicate percentage number of bootstrap iterations which support the tree at that particular point/ at each node of the phylogenetic tree.

Species close to each other in the phylogenetic tree are closely related. Based on *MatK* gene sequences, *P.caninus, P.otostegioides, P. barbatus* and *P. lanuginosus* have been shown to be closely related (Cluster A), while *P.ornatus*, *P.pseudomarrubioides* and *P.eagyptiacus* have

been grouped together (Cluster B). The phylogenetic tree also shows that *P.montanous* and *P.amboinicus* are closely related and form a monophyletic group (Cluster C). All the species share a distant common ancestor with *P.edulis*

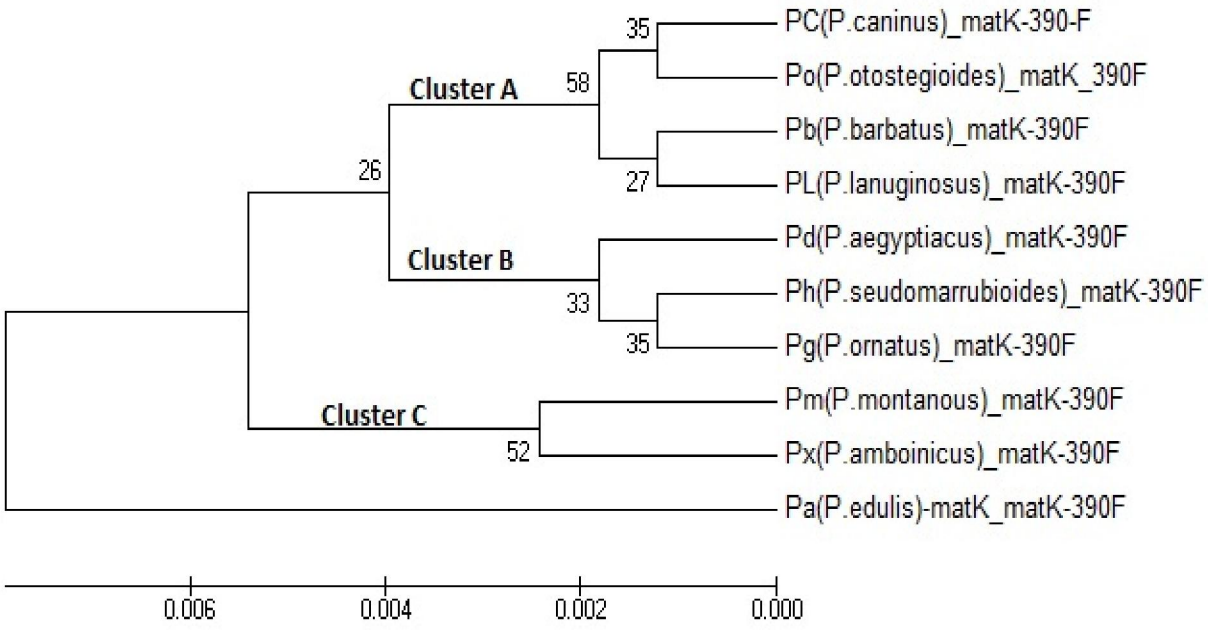


Figure 4.5: A phylogenetic tree for the ten *Plectranthus* species based on *MatK* gene sequences

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The following is the most likely phylogenetic tree created based on *Rbcl* gene sequences from the ten species showing various taxonomic relationships among the *Plectranthus* species under study. Based on the *Rbcl* gene sequences, *P. pseudomarrubioides, P.montanous, P.ornatus, P.amboinicus* and *P. edulis* have also been grouped together (Cluster D) while *P. caninus, P.lanuginosus, P.barbatus* and *P. otostegioides* have also been grouped together (Cluster E) and both groups are related to *P. aegyptiacus* which is grouped alone (Figure 4.6).

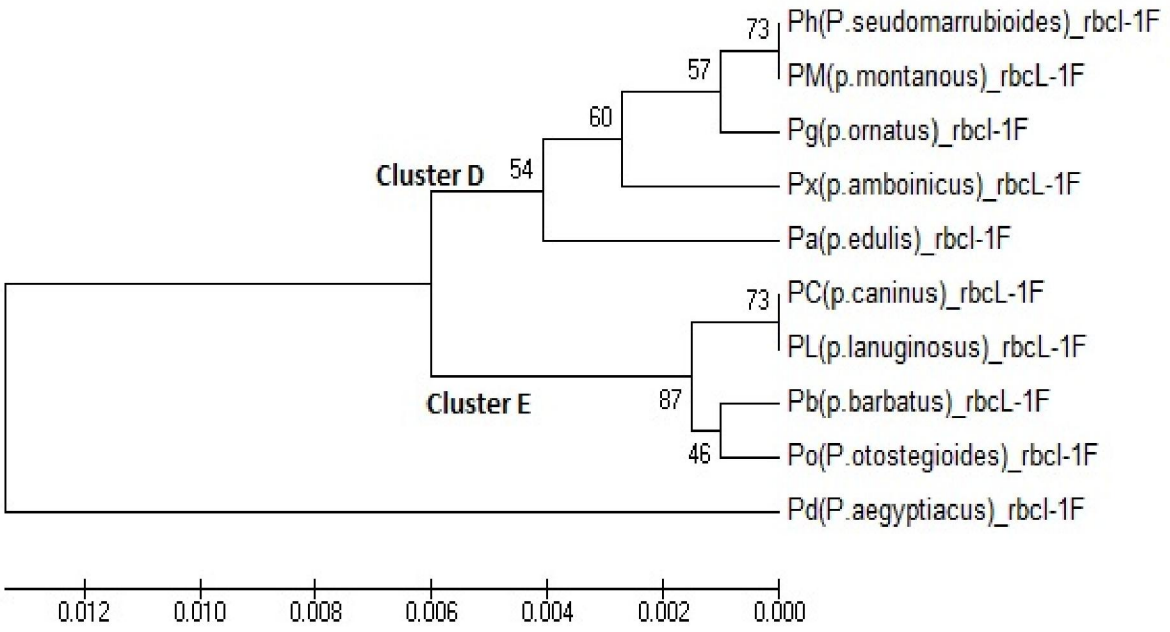


Figure 4.6: A phylogenetic tree for the ten *Plectranthus* species based on *Rbcl* gene sequences

Comparison of the two phylogenetic trees produced using the *MatK* genes and *Rbcl* genes has been presented in Figure 4.7. The two phylogenetic trees are strikingly similar as it is ndicated by the coloured circles. For example based on the *MatK* genes; *P. caninus*, *P. otostegioides, P. barbatus* and *P. lanuginosus* have been grouped together and all the four species form a monophyletic group (Cluster A). The same pattern can be observed in the phylogenetic tree created using the *Rbcl* genes (Cluster E). Again based on the *MatK* genes; *P. pseudomarrubioides*, *P. ornatus*, *P. montanous* and *P. amboinicus* have been showed to be closely related (Cluster B).

This close relationship of these species can also be observed in phylogenetic tree produced using the *Rbcl* genes (Cluster D). However based on *MatK* genes, *P.aegyptiacus* have been shown to be closely related to *P. pseudomarrubioides* and *P.ornatus* (Clade B) while based on *Rbcl* genes, *P.edulis* is closely related to *P.amboinicus, P.ornatus, P.pseudomarrubioides, P.ambonicus* and *P. montanous* (Clade D).

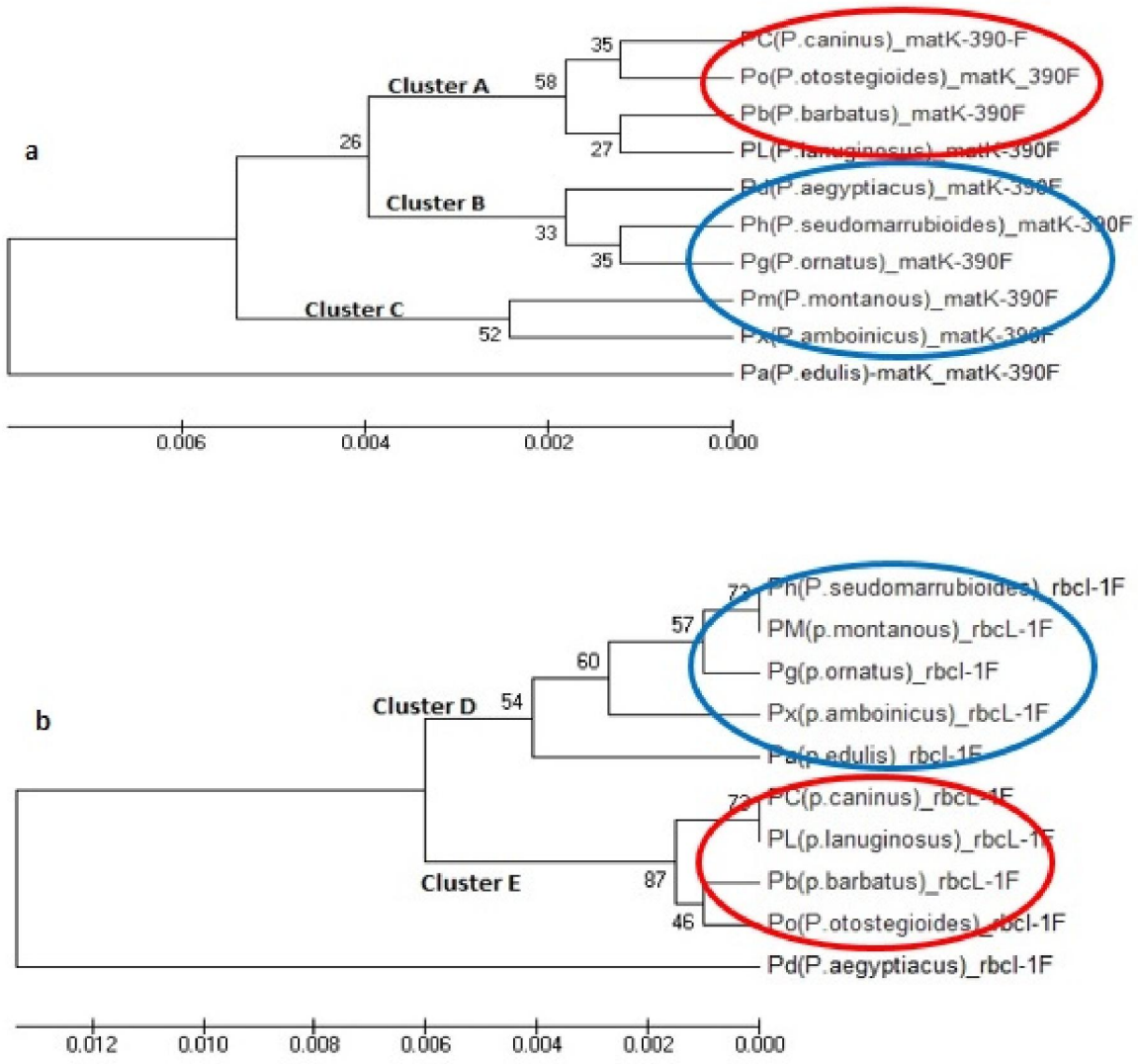


Figure 4.7: Comparison of *MatK* (a) and *Rbcl* (b) phylogenetic trees of the ten *Plectranthus* species

4.2.4. Comparison of the *Plectranthus* species genes with genbank sequences

The sequences from the two genes were subjected to Basic Local Alignment Search (BLAST); a sequence similarity search tool in the National Center for Biotechnology Information (NCBI) website to identify other similar sequences of *Plectranthus* species in the *Coleus* clade already deposited in NCBI genebank database for comparison with the sequences from the ten *Plectranthus* species. 18 *Rbcl* gene sequences and 26 *MatK* gene sequences of different

Plectranthus species were found to have been deposited in the genebank at NCBI (NCBI Nucleotide Database, 2016). From the *Plectranthus* species under study, only *P. barbatus*, *P. caninus* and *P. amboinicus MatK* and *Rbcl* genes genes were already deposited in the genbank. Not all *Plectranthus* species whose *MatK* and *Rbcl* genes have been deposited in the gene bank have complete names, some have only the genus names as seen in Figure 4.8 and 4.9. Comparison of the sequences from *Plectranthus* species already deposited in the NCBI and the sequences from the ten species investigated was done and phylogenetic trees were generated to show the phylogenetic position of the ten species among other species in the wider *Plectranthus* genus. The following most likely phylogenetic trees were constructed.

A comparative phylogenetic tree generated based on *MatK* gene sequences from the ten *Plectranthus* species and other 26 *MatK* genes sequences obtained from NCBI has been presented in Figure 4.8. *P.amboinicus* is one of the ten studied species and has been shown to be very closely related to *Plectranthus fruticosus* L'Herit., *Plectranthus zeylanicus* Benth. and *Plectranthus prostratus* Gürke while *P.montanous* which also form part of the ten species under investigation has been shown to be closely related to *Plectranthus mollis* (Aiton) Spreng. and *Plectranthus malabricus* (Benth.) R.H.Willemse.

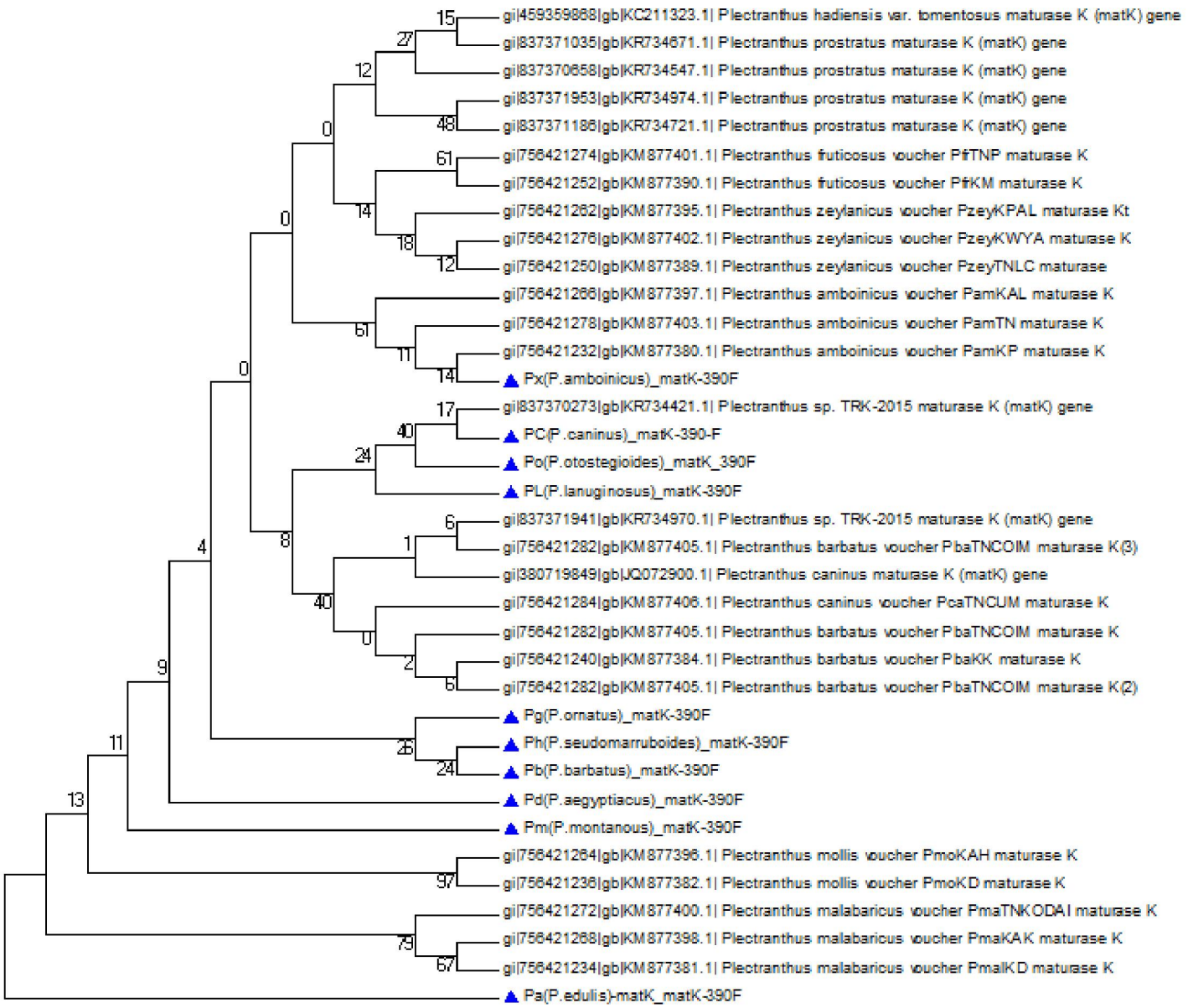


Figure 4.8: Comparison of current project *MatK* sequences with those obtained from genbank *Plectranthus* sequences

**The 10 species under study have been indicated with a blue triangular mark*

A comparative phylogenetic tree generated based on *Rbcl* gene sequences from the ten *Plectranthus* species and 18 *Rbcl* sequences obtained from NCBI (Figure 4.9). *P. ornatus* is one of the ten species investigated and is shown to be closely related to *Plectranthus parviflorus* Wild. and *P. amicorum* while *P. edulis, P. montanous* and *P. pseudomarrubioides* which also form part of the ten studied species have been shown to be closely related to *P. prostratus* and lastly *P. aegyptiacus* and *P. hadiensis* have been shown to be closely related and form a monophyletic group.

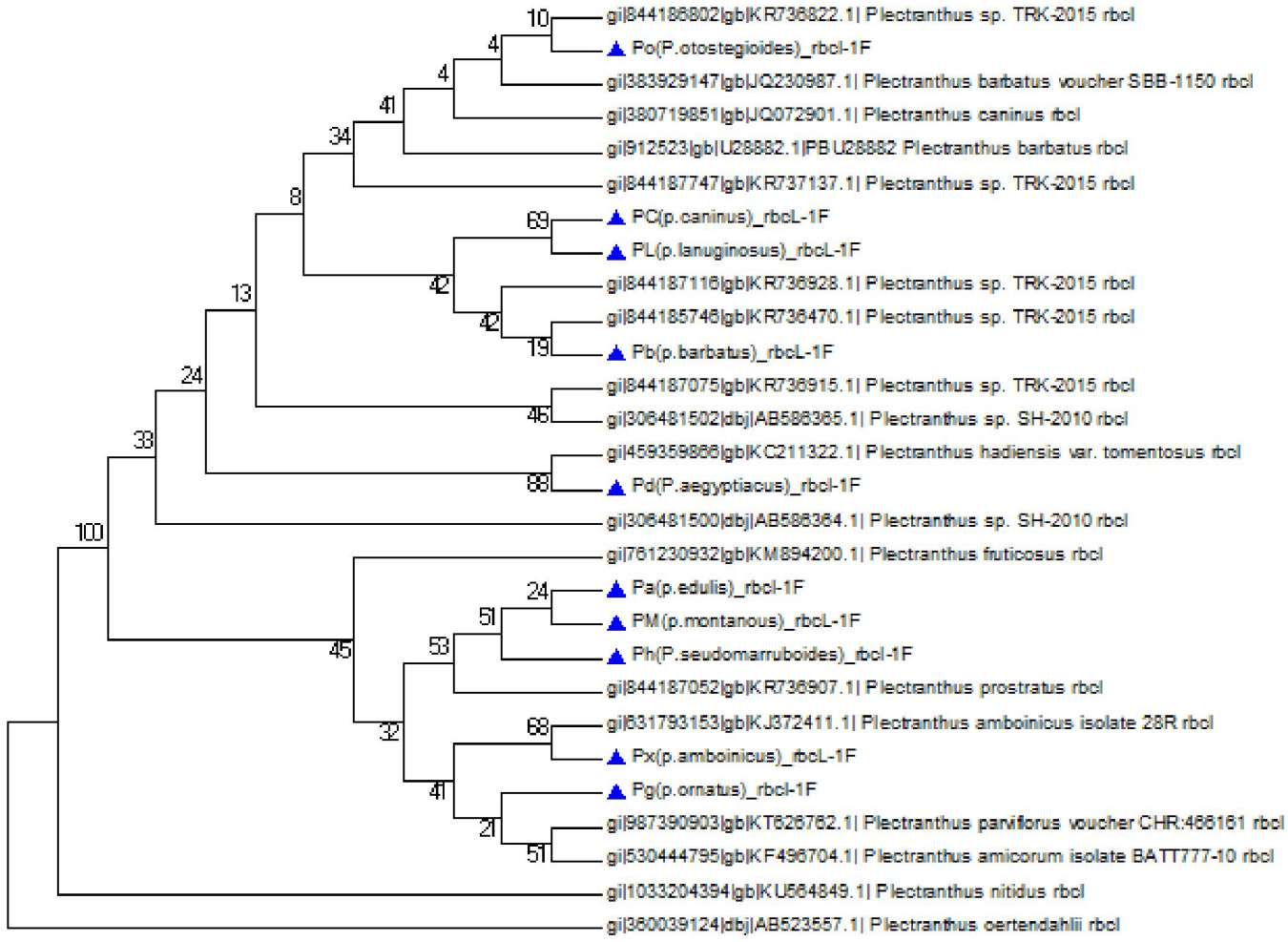


Figure 4.9: Comparison of current project *Rbcl* sequences with those from genbank *Plectranthus* sequences

**The 10 species under study have been indicated with a blue triangular mark*

4.3. Leaf micromorphology of *Plectranthus* species

4.3.1. Stomatal distribution in *Plectranthus* species

The number of stomata counted on the abaxial and adaxial surfaces of the leaves of the ten *Plectranthus* species investigated have been tabulated on Table 4.1.

Table 4.1: Stomata distribution in the ten *Plectranthus* species

		Number of stomata per magnification field (mg= x 400) [Fixed Eyepiece (x 10) * Adjustible Objective lens (x 40)]			
Species	Place collected	Average No. of stomata on the abaxial (lower) surface	Standard Error of the Mean	No. of stomata on the adaxial (upper) surface	Standard Error of the Mean
P. caninus	Ngong'	35	±0.23	25	±0.81
P. aegyptiacus	Murang'a	50	±0.32	36	±0.26
P. edulis	Transnzoia	47	±0.43	35	±0.29
P.pseudomarrubioides	Naivasha	36	±0.58	25	±0.44
P. barbatus	Nairobi	45	±0.11	39	±0.71
P. lanuginosus	Nairobi	58	±0.76	49	±0.90
P. otostegioides	Olorgesailie	28	±0.52	20	±0.53
P.montanous	Makueni	28	±0.66	23	±0.39
P. ornatus	Trans-Nzoia	35	±0.27	27	±0.18
P .amboinicus	Nairobi	45	±0.92	33	±0.83

Average number of stomata on both sides was highest in *P. barbatus*, *P. amboinicus, P.aegyptiacus, P. lanuginosus* and *P. edulis*. Fewer numbers of stomata were observed in *P. pseudomarrubioides, P. otostegioides* and *P. montanous.* In all the other species, fewer numbers of stomata was observed in the adaxial surface compared to the abaxial surface .

4.3.2. Leaf micromorphology of *Plectranthus* species

Variations in leaf anatomical characters obtained from the leaves of the ten *Plectranthus* species are tabulated in Table 4.2. The characters include: Nature of the leaf, stomata occurrence, stomata type, epidermal cell type, trichome type, glandular trichomes, nature of the mesophyll and nature of the palisade layer.

Table 4.2: Leaf anatomical characters in the ten *Plectranthus* species

Species	Nature of the leaf	Stomata occurence	Stomata type	Epidermal cell type	Trichome type	Gradular trichomes	Mesophyll	Palisade layer
P.caninus	Succulent	Ab/Ad	anomocytic	Polygonal	Long- stalked	Present	dorsiventral	Long columnar cells
P.aegyptiacus	Succulent	Ab/Ad	anomocytic	Sinuous	Short stalked	Present	homogenous	Inconspicuous
P.edulis	Non succulent	Ab/Ad	anomocytic	Polygonal	Short stalked	Absent	dorsivental	Long columnar cells
P.pseudomarrubioides	Succulent	Ab/Ad	anomocytic	Sinuous	Short stalked	Present	homogenous	Inconspicuous
P.barbatus	Succulent	Ab/Ad	anomocytic	Polygonal	Capitate	Present	dorsiventral	Long columnar cells
P.lanuginosus	Succulent	Ab/Ad	anomocytic	Polygonal	Capitate	Present	homogenous	Inconspicuous
P. otostegioides	Succulent	Ab/Ad	anomocytic	Sinuous	Short-stalked	Present	homogenous	Inconspicuous
P.montanous	Succulent	Ab/Ad	anomocytic	Sinuous	Short-stalked	Present	homogenous	Inconspicuous
P.ornatus	Succulent	Ab/Ad	anomocytic	Polygonal	long-stalked	Present	dorsivental	Long columnar cells
P.amboinicus	Succulent	Ab/Ad	anomocytic	Sinuous	Short stalked	Absent	homogenous	Inconspicuous

Key: *Sinuous:* containing many curved edges, *Polygonal:* resembling a polygon, *Anomocytic:* surrounded by more than four subsidiary cells, *Diacytic:* surrounded by two subsidiary cells, *Tetracytic:* surrounded by four cells, *Homogeneous mesophyll:* a mesophyll whose layers are hard to differentiate, *Dorsiventral mesophyll*: A mesophyll with distinct dorsal and ventral surfaces, *Capitate;* A trichome culminating in a compact head, *Ab/Ad*: Abaxial/Adaxial leaf surface.

Below are some images supporting the above stomatal distribution and leaf anatomy data.

Upper leaf surface of *P.caninus* showing stomata, which are anomocytic; stomata are surrounded by more than four subsidiary cells (mg= ×400) (Figure 4.10).

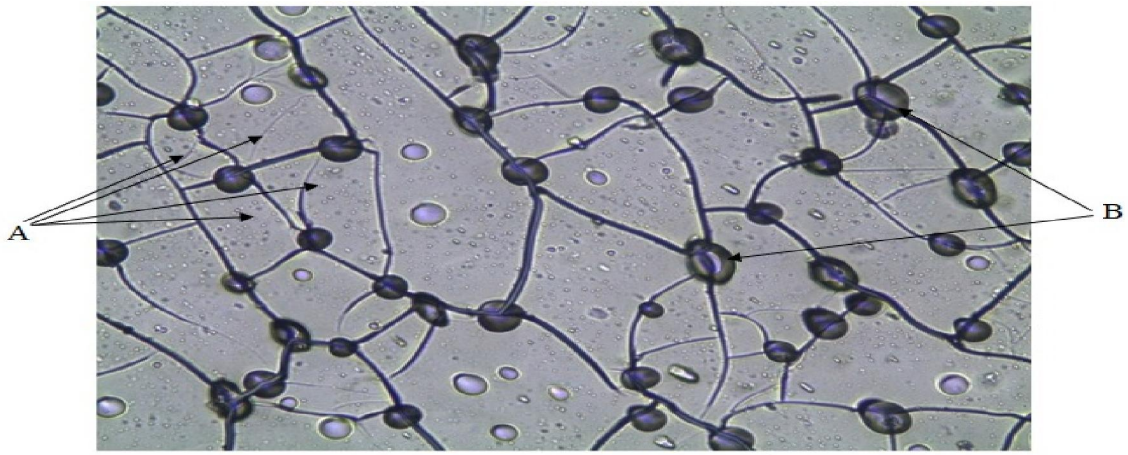


Figure 4.10: Adaxial surface of *P.caninus* leaf

A: Subsidiary cells, **B**: Anomocytic stomata

Lower surface of *P. pseudomarrubioides* showing anomocytic stomata (A) (mg=×400)

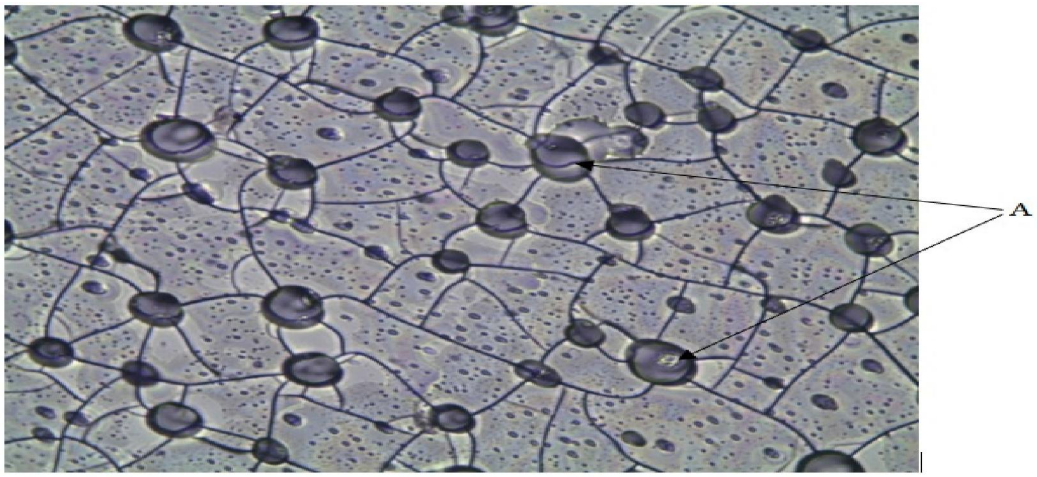


Figure 4.11: Abaxial surface of *P.pseudomarrubioides* leaf

A: Anomocytic stomata

Transverse section of *P.lanuginosus* leaf showing homogenous mesophyll (**A**), sinuous epidermal cells (**B**) and long stalked trichomes (**C**) (mg=×100) (Figure 4.12).

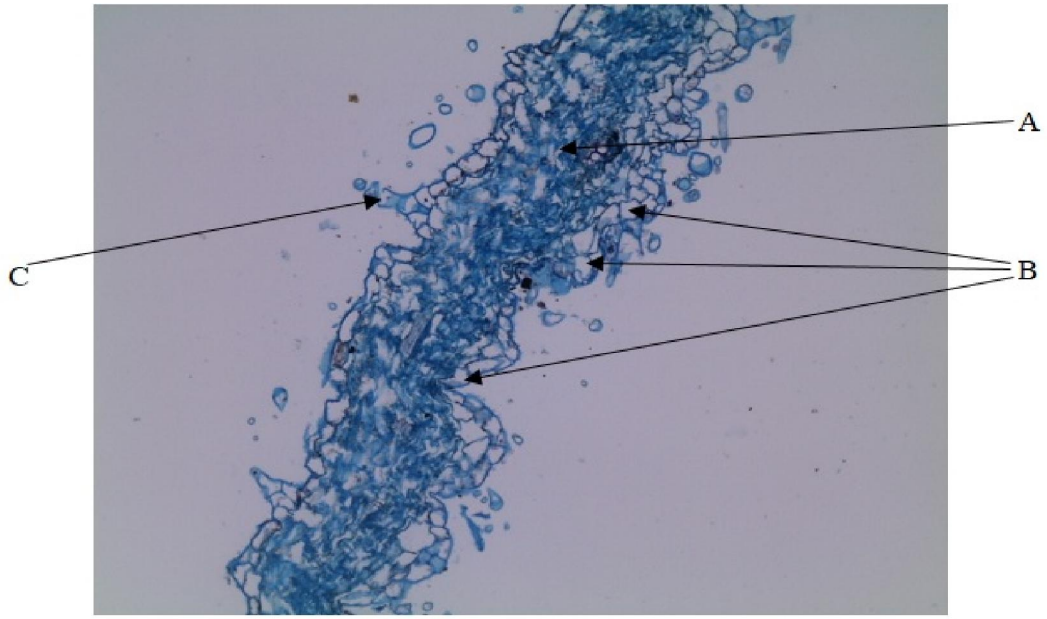


Figure 4.12: Transverse section of *P.lanuginosus* leaf

A: homogenous mesophyll, **B:** sinuous epidermal cells, **C:** long stalked trichomes

The following is an image of *P.ornatus* showing long columnar cells, long stalked trichomes, dorsiventral mesophyll and polygonal epidermal cells (Figure 4.13). Such a pattern was also observed in *P.edulis*, *P.barbatus* and *P.caninus.*

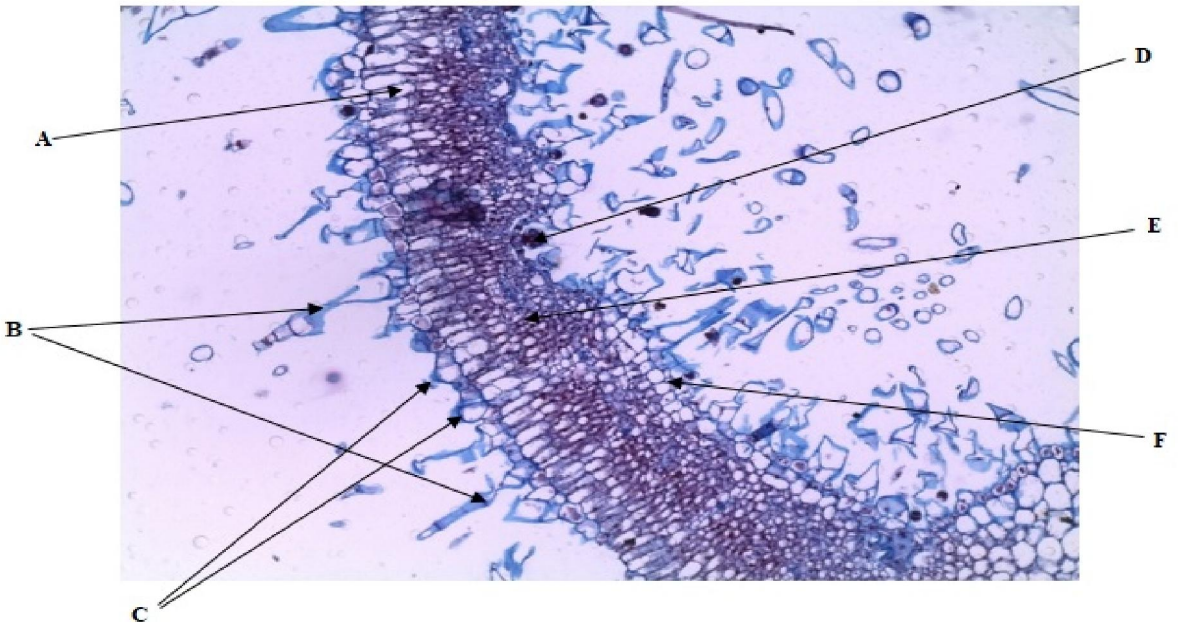


Figure 4.13: Transverse section of *P.ornatus* leaf

A: Long columnar cells in the palisade layer, **B**: Long stalked trichomes, **C**: Non-glandular trichomes, **D**: Capitate glands, **D**: Dorsiventral mesophyll, **E**: Dorsiventral Mesophyll, **F:** Polygonal epidermal cells

Transverse section of *P.barbatus* is presented in Figure 4.14. Apart from being observed in *P.barbatus*, polygonal epidermal cells, dorsiventral mesophyll has been observed in *P.caninus, P.edulis* and *P.ornatus*

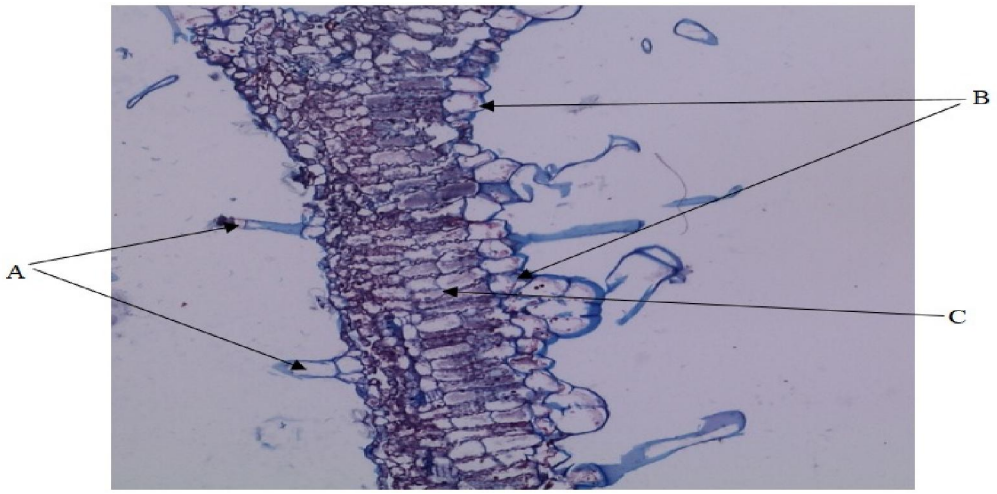


Figure 4.14: Transverse section of *P.barbatus* leaf

A: capitate trichomes, **B**: polygonal epidermal cells, **C**: dorsiventral mesophyll

Transverse section of *P.edulis* shows the leaf has dorsiventral mesophyll (**A**), polygonal epidermal cells (**B**), long columnar cells (**C**) and lacks trichomes on the epidermis (Figure 4.15).

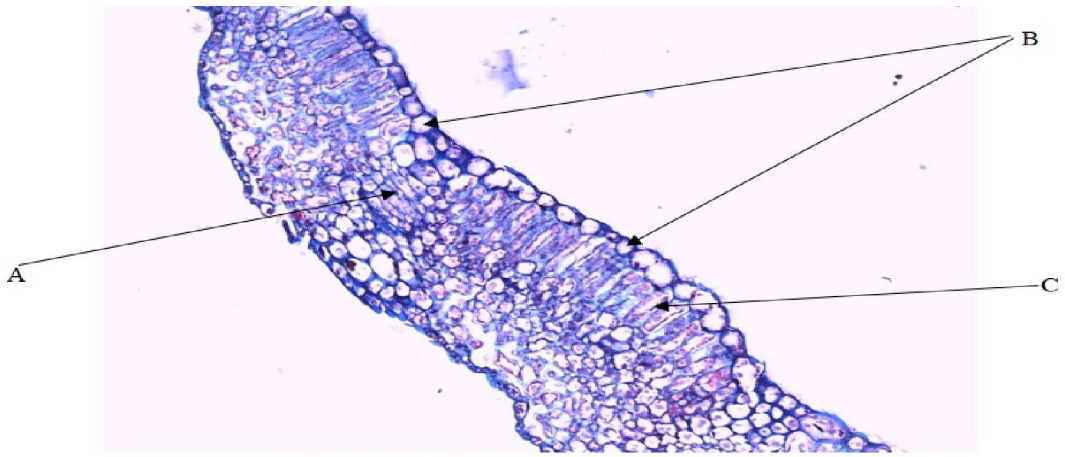


Figure 4.15: Transverse section of *P.edulis* leaf

Below is presentation of trichomes observed in the ten *Plectranthus* species (Figure 4.16).

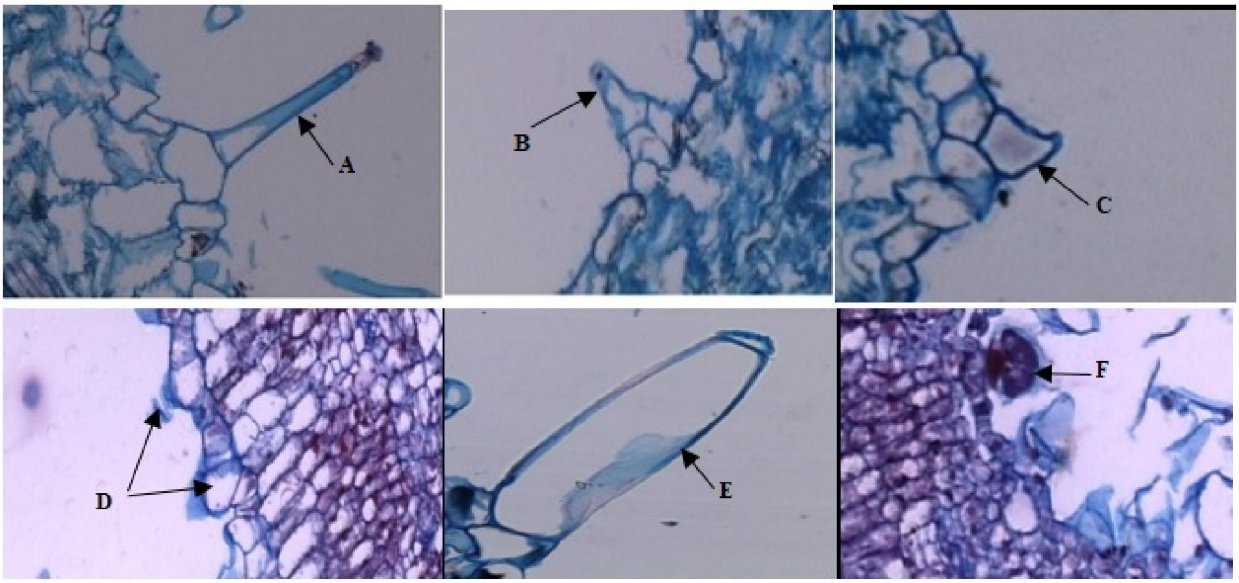


Figure 4.16: Trichomes identified in *Plectranthus* species

A: Long stalked glandular trichome (in *P.caninus and P.ornatus*), **B:** short stalked glandular trichome (in *P.aegyptiacus, P.edulis, P. pseudomarrubioides, P.otostegioides, P.montanous, P.ambonicus*), **C:** Capitate non-stalked trichome (in *P.ornatus*), **D:** Non-glandular trichomes (in *P.ornatus*), **E:** Capitate glandular trichome (in *P. lanugonosus* and *P. barbatus*), **F:** Capitate glands (in *P.ornatus*).

Leaf anatomical characters which contributed to most of the differences in the ten *Plectranthus* investigated include: epidermal cell type which was either polygonal (Figure 4.13) or sinuous (Figure 4.12), trichome type which ranged from being long stalked, short stalked, glandular, non glandular to capitate (Figure 4.16), nature of the leaf mesophyll which was either dorsiventral (where upper epidermis, palisade layer, spongy mesophyll and lower epidermis were easily identified as distinct)(Figure 4.14) or homogeneous (whereby the various layers of the leaf mesophyll were indiscreet and not easily identified)(Figure 4.13). For the *Plectranthus* species with dorsiventral mesophyll, the palisade layer could easily be identified bearing the usual long columnar cells (Figure 4.14 and Figure 4.15) while for those with homogeneous mesophyll, the palisade layer was inconspicous just like the other layers of that homogenous mesophyll.

4.3.3. Dendrogram based on the leaf micromorphology data

The leaf micromorphology data obtained from the current study was subjected to hierarchical cluster analysis. Character states were assigned values and scored in a matrix, the following matrix was subjected to cluster analysis.

Table 4.3: Leaf micromorphology character states matrix of the ten *Plectranthus* species

	Leaf micromorphology characters from the ten *Plectranthus* species										
Plectranthus species	Succulent leaf	stomata distribution	stomata type	Epidermal Cell type	Trichome type	Glandular Trichomes	Nature of mesophyll	Palisade layer	Stomata No. (adaxial)	Stomata No.(abaxial)	
P.caninus	1.00	3.00	1.00	1.00	2.00	1.00	1.00	1.00	2.00	3.00	Character state scores/values
P.aegyptiacus	1.00	3.00	1.00	2.00	2.00	1.00	2.00	2.00	3.00	4.00	
P.edulis	2.00	3.00	1.00	1.00	2.00	2.00	1.00	1.00	3.00	4.00	
P.seudomarrubioides	1.00	3.00	1.00	2.00	2.00	1.00	2.00	2.00	3.00	3.00	
P.barbatus	1.00	3.00	1.00	1.00	1.00 & 3.00	1.00	1.00	1.00	3.00	4.00	
P.lanuginosus	1.00	3.00	1.00	1.00	1.00 & 3.00	1.00	2.00	1.00	4.00	5.00	
P.otostegioides	1.00	3.00	1.00	2.00	2.00	1.00	2.00	2.00	2.00	2.00	
P.montanous	1.00	3.00	1.00	2.00	2.00	1.00	2.00	2.00	2.00	2.00	
P.ornatus	1.00	3.00	1.00	1.00	1.00 & 3.00	3.00	1.00	1.00	2.00	3.00	
P.amboinicus	1.00	3.00	1.00	2.00	2.00	2.00	2.00	2.00	3.00	4.00	

Succulent leaf: 1(present), 2 (absent), *Stomata occurrence:*1(adaxial surface), 2(Abaxial surface), 3(both surfaces), *Stomata type:* 1(anomocytic), *Epidermal cell type:* 1(sinuous), 2(polygonal), *Trichome type:* 1(long stalked), 2(short stalked), 3(capitate), *Grandular tichomes:* 1(present), 2 (absent), 3 (both glandular and non glandular trichomes present), *Mesophyll:*1(homogenous), 2(dorsiventral), *Palisade layer:* 1(normal long columnar cells), 2(incospicous), *Stomata number on the adaxial surface*: 1(<20 stomata), 2(20-30 stomata), 3(30-40 stomata), 4(40-50 stomata),5(>50 stomata), *Stomata number on the abaxial surface:* 1(<20 stomata), 2(20-30 stomata), 3(30-40 stomata), 4(40-50 stomata),5(>50 stomata).

Hierarchical cluster analysis of the leaf micromorphology character state matrix in Table 4.3 in SPSS led to the following UPGMA dendrogram (Figure 4.17). The dendrogram in Figure 4.17 shows two main groups. *P.caninus, P.ornatus, Potostegioides, P.montanous* and *P. pseudomarrubioides* together in one group (Cluster F) while *P. aegyptiacus, P.amboinicus, P.edulis, P.barbatus* and *P.lanuginosus* have been placed in another group (Cluster G). The inner branches of the dendrogram show that *P. caninus* is similar to *P.ornatus. P.otostegioides*, *P.montanous* and *P. pseudomarrubioides* are likewise similar hence have been grouped together. *P.aegyptiacus* has also been placed close to *P.ambonicus* and lastly *P.edulis, P.barbatus* and *P.lanuginosus* have been grouped together as well.

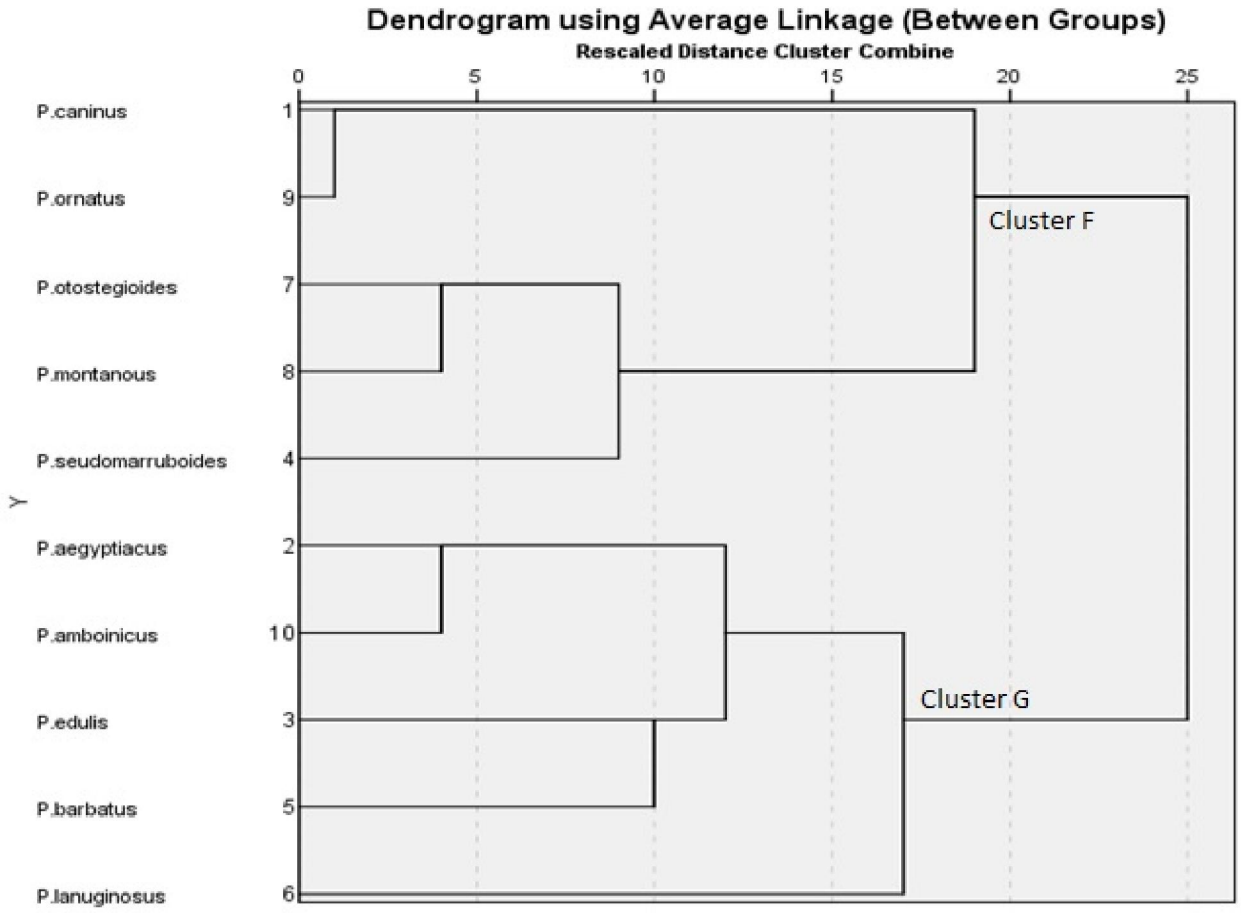


Figure 4.17: Leaf micromorphology dendrogram

4.3.4. Dendrogram based on morphological characters described in the FTEA

Using gross morphological characters of the ten *Plectranthus* species, the following character state matrix (Table 4.4) was created which was analysed through hierarchical cluster analysis.

Table 4.4: Gross morphology character state matrix of the ten *Plectranthus* species

	Gross morphological characters from the ten *Plectranthus* species																			
Pletranthus species	Succulent leaf	Height	Inflorescence	Cymes	Pedicel length	Calyx length	Calyx nature	Corolla length	Corolla colour	Leaf apex	Bracts	Nutlets	Staminal filaments	Leaf blade	Petiole	Stems	Stems colour	Egrandular hairs	Stems shape	Character state scores/values
P.barbatus	1.00	4.00	2.00	2.00	3.00	3.00	1.00	3.00	4.00	1.00	1.00	2.00	1.00	1.00	3.00	1.00	1.00	1.00	1.00	
P.caninus	1.00	2.00	1.00	1.00	1.00	3.00	1.00	2.00	1.00	1.00	2.00	3.00	1.00	1.00	1.00	2.00	3.00	2.00	1.00	
P.montanous	1.00	1.00	3.00	3.00	1.00	1.00	2.00	1.00	1.00	1.00	3.00	1.00	2.00	1.00	4.00	1.00	2.00	3.00	1.00	
P.otostegioides	1.00	2.00	1.00	1.00	3.00	3.00	1.00	2.00	2.00	1.00	4.00	1.00	1.00	1.00	1.00	2.00	3.00	2.00	1.00	
P.lanuginosus	1.00	3.00	2.00	1.00	2.00	2.00	3.00	1.00	3.00	2.00	1.00	2.00	1.00	2.00	3.00	2.00	3.00	2.00	1.00	
P.seudomarruboides	1.00	3.00	2.00	2.00	1.00	1.00	3.00	2.00	4.00	2.00	5.00	1.00	2.00	3.00	2.00	1.00	1.00	3.00	1.00	
P.edulis	2.00	4.00	1.00	1.00	2.00	2.00	1.00	1.00	4.00	1.00	1.00	2.00	1.00	1.00	2.00	2.00	3.00	4.00	1.00	
P.ornatus	1.00	1.00	1.00	1.00	1.00	1.00	1.00	3.00	4.00	1.00	2.00	3.00	1.00	1.00	1.00	2.00	3.00	1.00	1.00	
P.aegyptiacus	1.00	1.00	3.00	3.00	3.00	2.00	3.00	2.00	2.00	3.00	2.00	1.00	1.00	2.00	2.00	2.00	1.00	1.00	1.00	
P.amboinicus	1.00	2.00	2.00	2.00	1.00	2.00	4.00	2.00	2.00	1.00	1.00	1.00	1.00	1.00	2.00	1.00	1.00	3.00	1.00	

Succulent leaf: 1(present), 2(absent), *Height:*1(<0.3 m), 2(0.3-0.5 m), 3(0.5-1 m),4(>1 m), *Inflorescence:* 1(with <10 flowered verticils), 2(with 10-20 flowered verticils),3(with >20 flowered verticils), *Cymes:*1(<5 flowered), 2(5-10 flowered),3(>10 flowered), *Pedicel length:*1(<3 mm long), 2(3-6 mm long), 3(>6 mm long),*Calyx length:* 1(<2 mm long), 2(2-4 mm long), 3(>4 mm long), Nature of calyx: 1(pubescent), 2(tomentose), 3(villose), 4(pubescent to villose),*Corolla length:* 1(<10 mm long), 2(10-20 mm long), 3(>30 mm long), *Corolla colour:* 1(Blue to purple with white marks), 2(white with white marks), 3(pale blue to lilac), 4(blue with purple marks), *Leaf apex:*1(obtuse to rounded), 2(obtuse), 3(acute to rounded), *Bracts:* 1(ovate,apiculate), 2(ovate), 3(ovate to elliptic),4(obovate to elliptic), 5(broadly elliptic), *Nutlets:*1(brown with reddish dots), 2(brown with dark dots), 3(dark grey with dark dots), *Staminal filaments:* 1(fused), 2(free), Leaf blade:1(elliptic,ovate,obovate), 2(triangular),3(trullate,rhombic), *Petiole length:* 1(<10 mm long), 2(10-20 mm long), 3(20-50 mm long),4(epetiolate/sessile), *Stem:*1(succulent), 2(non succulent/soft wooded), *Stem colour:* 1(sometimes purplish above), 2(pinkish to dark red), 3(unknown), *Eglandular hairs:*1(antrose and patent),2(patent), 3(retrose and patent), 4(unknown), *Stem shape:*1 (rounded and quadrangular)

After hierarchical cluster analysis of the gross morphology character state matrix, the following UPGMA dendrogram was constructed (Figure 4.18). It shows the existing taxonomic relationships of the *Plectranthus* species based on gross morphological characters as described by Paton *et al*., (2009).

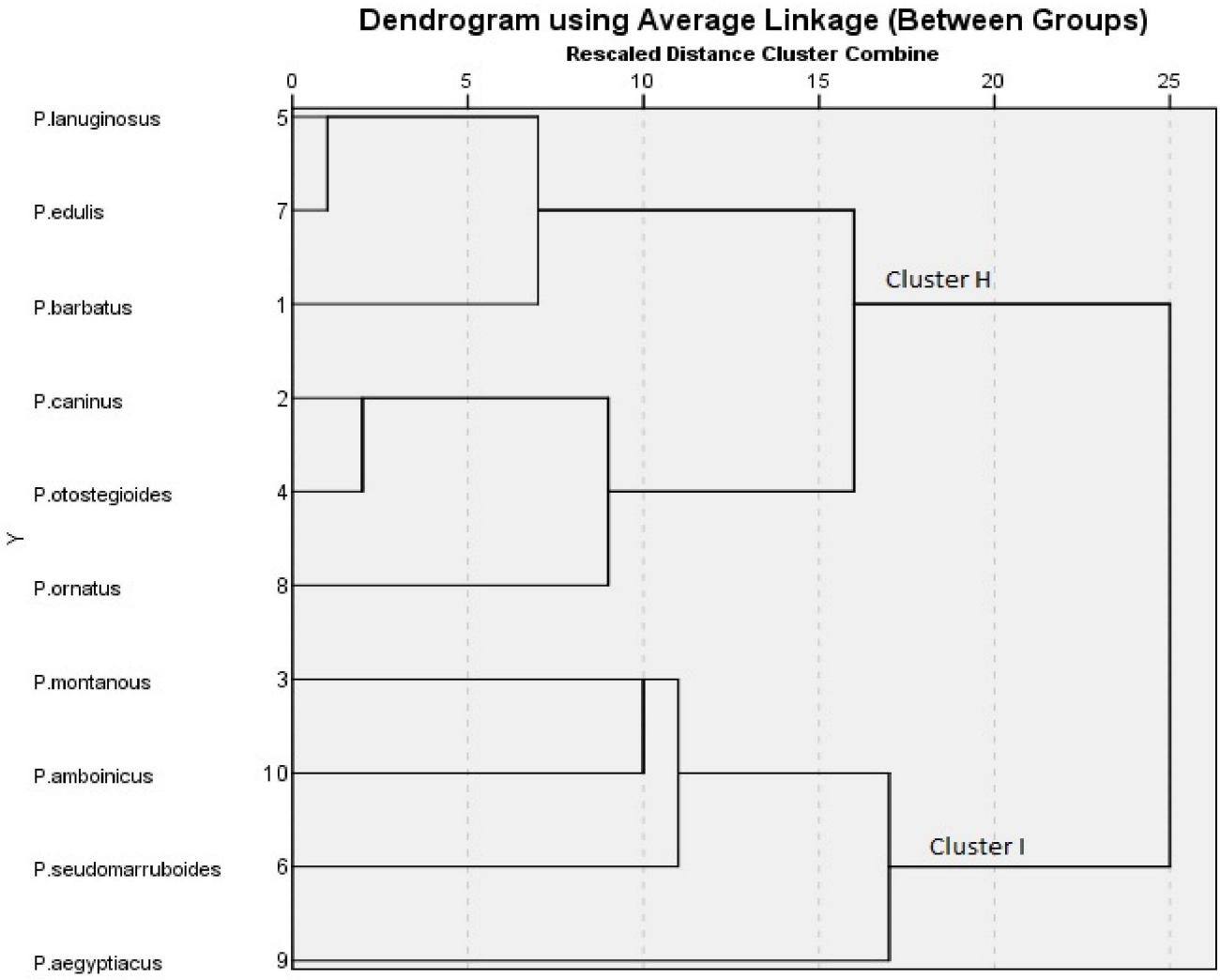


Figure 4.18: Dendrogram showing taxonomic relationships of the ten *Plectranthus* species according to FTEA

Two main clusters/groups are evident from the dendrogram above which occur at about the same horizontal distance. One cluster (H) contains *P. lanuginosus, P. edulis, P. barbatus, P. caninus*, *P. otostegioides* and *P. ornatus*. The other cluster (I) contains *P. montanous, P. amboinicus, P. pseudomarrubioides* and *P. aegyptiacus*. Cluster H further shows *P. lanuginosus, P. edulis, P. barbatus* have been grouped together and *P. caninus*, *P. otostegioides* and *P. ornatus* have also been grouped together as well. This implies that members within each cluster are very close morphologically (Figure 4.18).

4.3.5. Comparison of leaf micromorphology dendrogram with *MatK* and *Rbcl* phylogenetic trees

Comparison of the leaf micromorphology dendrogram with the *MatK* and *Rbcl* phylogenetic trees was done to determine whether the two have any classification similarity. Similarities which can be inferred from the leaf micromorphology dendrogram and the molecular phylogenetic trees include: both the dendrogram and the phylogenetic trees have placed *P.barbatus* and *P.lanuginosus* in the same group. Again both the dendrogram and the molecular phylogenetic trees have placed *P. pseudomarrubioides* and *P. montanous* and *P.ornatus* together in the same group (Figure 4.19).

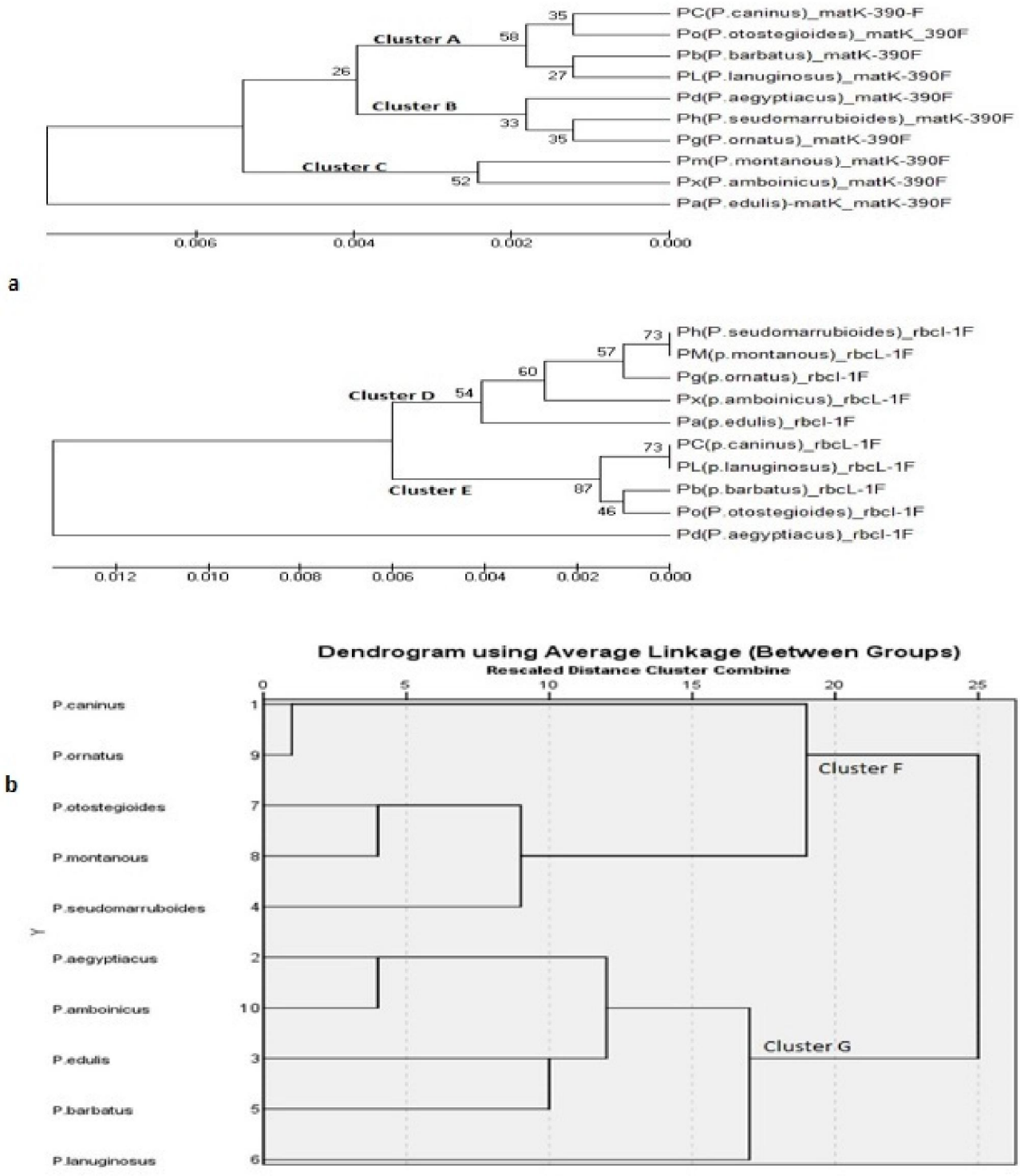


Figure 4.19: *MatK* and *Rbcl* phylogenetic trees (**a**) vs leaf micromorphology dendrogram (**b**)

4.4. Antimicrobial activity of *Plectranthus* species

4.4.1. Disc diffusion results

Dichloromethane/methanol [DCM: MeOH (1:1)] crude organic extract from each of the 10 species was subjected to antimicrobial activity through disc diffusion and the results are presented in Figure 4.20. It was observed that DCM: Methanol crude extracts from all the ten species investigated inhibited the multiplication of MRSA. Comparing all the species, highest inhibition zones were observed in 200 mg/ml and 100 mg/ml of *P. lanuginosus* which were 15 mm and 12.3 mm respectively and in 200 mg/ml and 100 mg/ml of *P. barbatus* which were 13 mm and 10.3 respectively, average inhibition zone for the positive control [Amoxicillin (50 mg/ml)] was 14 mm while the negative control (1% DMSO) did not inhibit growth of MRSA.

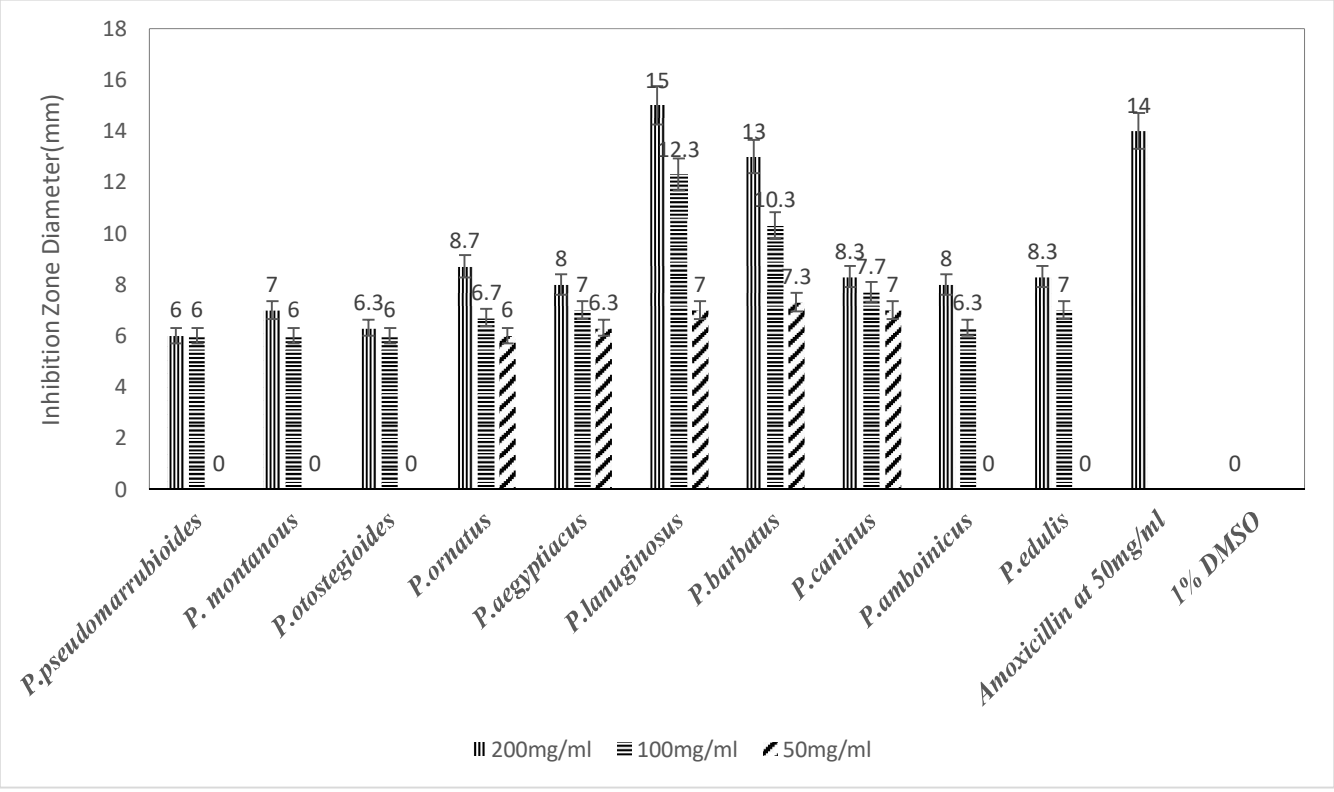


Figure 4.20: Growth inhibitions of MRSA by DCM-MeOH crude extracts of *Plectranthus* species at 200, 100 and 50 mg/ml

All the species investigated inhibited the growth of *B.cereus.* Among the ten species, highest growth inhibitions of *B.cereus* were observed in 200 mg/ml and 100 mg/ml of *P.otostegioides* which were 11.7 mm and 9.7 mm respectively, 200 mg/ml and 100 mg/ml of *P.lanuginosus* which were 11.7 mm and 10.7 mm respectively and 200 mg/ml and 100 mg/ml of *P.barbatus* which were 12.3 mm and 8.7 mm respectively, average inhibition zone for the positive control [Amoxicillin (50 mg/ml)] was 15 mm while the negative control (1% DMSO) did not inhibit growth of *B.cereus* (Figure 4.21).

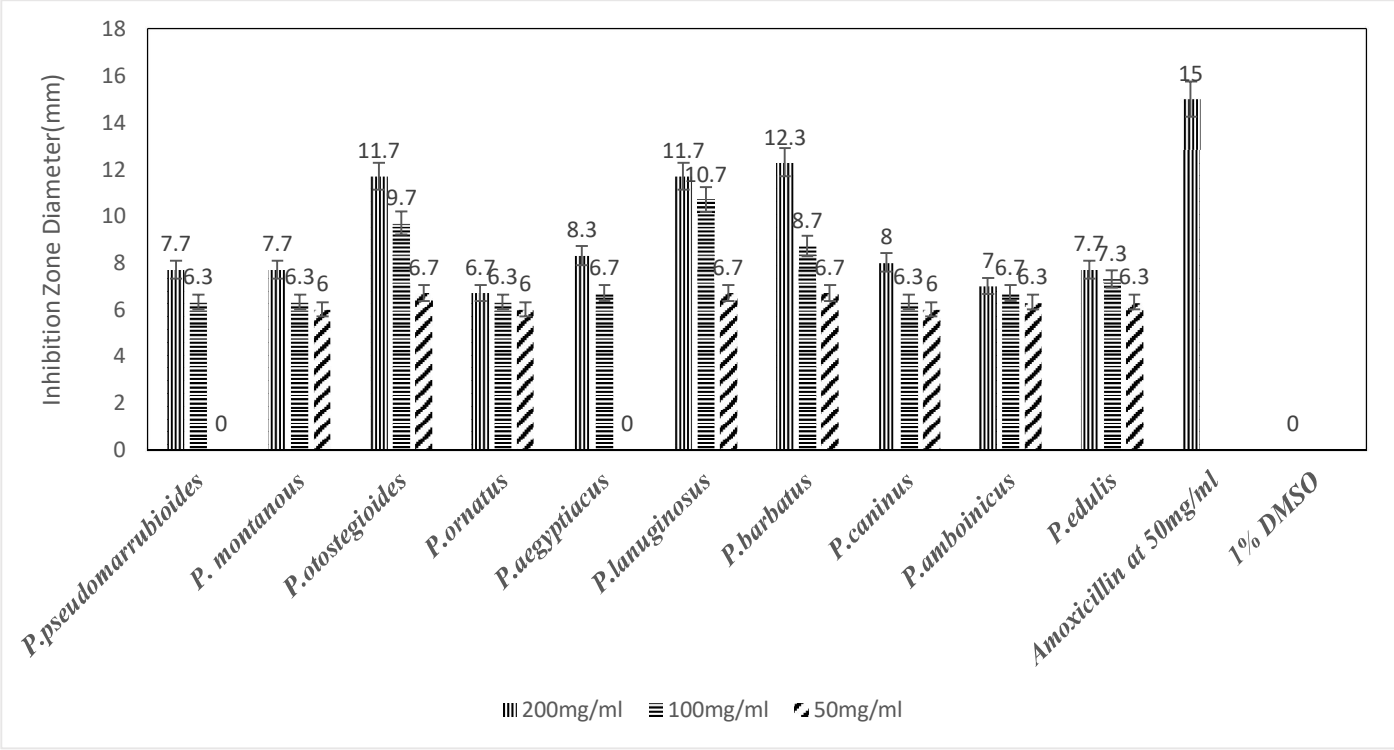


Figure 4.21: Growth inhibitions of *B.cereus* by DCM-MeOH crude extracts of *Plectranthus* species at 200, 100 and 50 mg/ml

It was observed that only the DCM: Methanol crude extracts from *P.barbatus* and *P.edulis* were able to inhibit the multiplication of *E. coli*. 200 mg/ml and 100 mg/ml of *P.barbatus* exhibited average inhibition zones of 9 mm and 7.7 mm respectively while 200 mg/ml and 100 mg/ml of *P.edulis* exhibited average inhibition zones of 8.3 mm and 6.3 mm respectively against *E.coli.* The other species did not inhibit the growth of *E.coli*, average inhibition zone for the positive control [Amoxicillin (50 mg/ml)] was 25 mm while the negative control (1% DMSO) did not inhibit growth of *E.coli* (Figure 4.22).

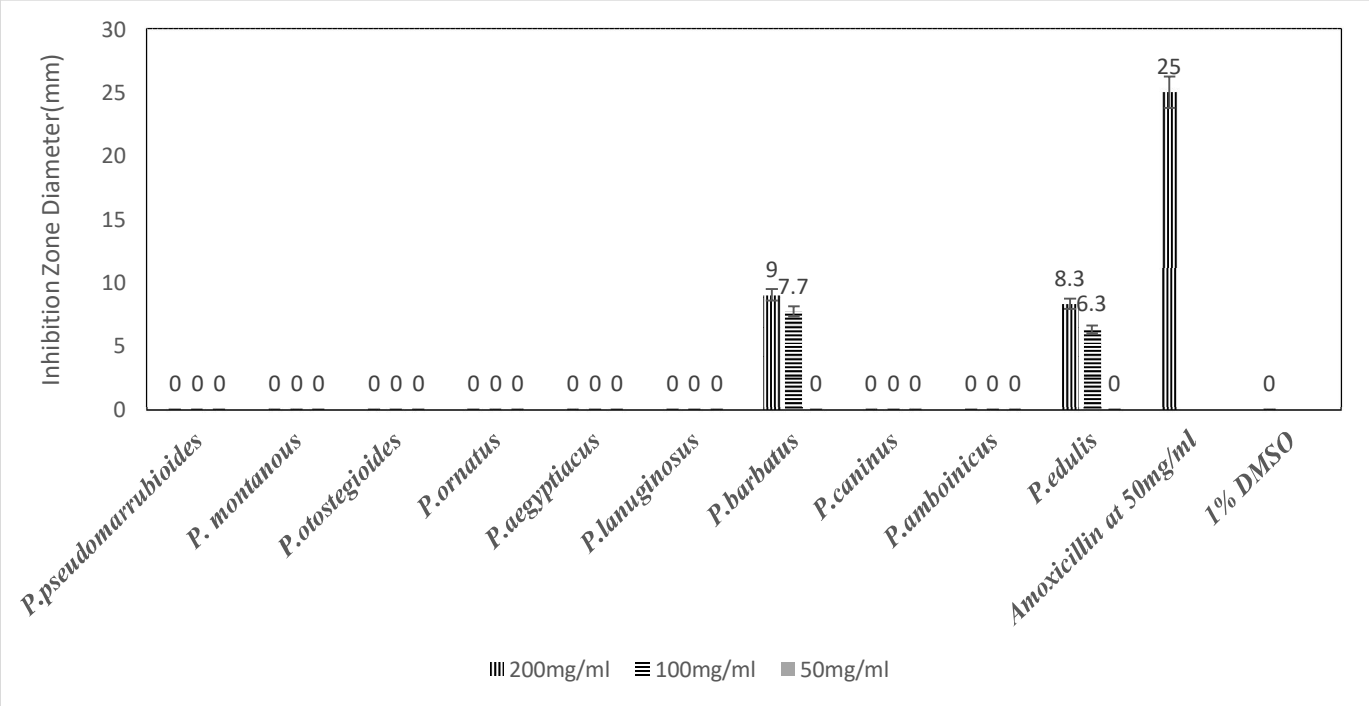


Figure 4.22: Growth inhibitions of *E.coli* by DCM-MeOH crude extracts of *Plectranthus* species at 200, 100 and 50 mg/ml

It was observed that all the species except *P.caninus* inhibited the multiplication of *C.albicans.* Comparing the ten species, highest growth inhibitions against *C.albicans* were observed in 200 mg/ml of *P. barabatus, P. ornatus, P. aegyptiacus, P. amboinicus* and *P. otostegioides* which were 13 mm, 12 mm, 10.3 mm, 10.3 mm and 10.3 mm respectively. Average inhibition zone for the positive control [ketoconazole (40 mg/ml)] was 13 mm while the negative control (1% DMSO) did not inhibit growth of *C.albicans* (Figure 4.23)

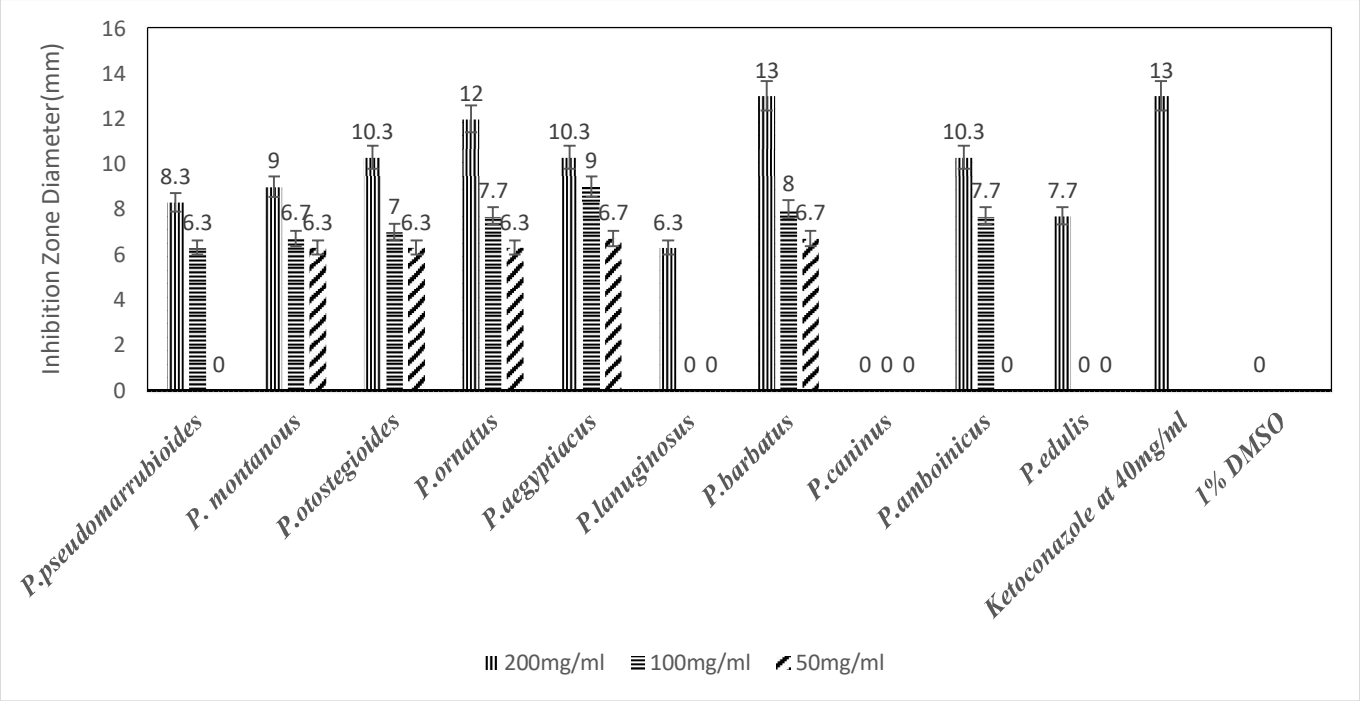


Figure 4.23: Growth inhibitions of *C.albicans* by DCM-MeOH crude extracts of *Plectranthus* species at 200, 100 and 50 mg/ml

P.montanous, P.otostegioides, P.aegyptiacus, P.lanuginosus, P.barbatus, P.amboinicus and *P.edulis* inhibited the multiplication of *A.niger*. *P. pseudomarrubioides, P.ornatus* and *P. caninus* did not inhibit the growth of *A.niger*. *P.lanuginosus* and *P. barbatus* exhibited higher inhibition zones against *A.niger* compared to the rest of the species. Average inhibition zones for 200 mg/ml *P. laniginosus* and *P.barbatus* were 13.3 mm and 13.7 mm respectively. Both ketoconazole (40mg/ml) and 1% DMSO did not inhibit the growth of *A.niger* (Figure 4.24).

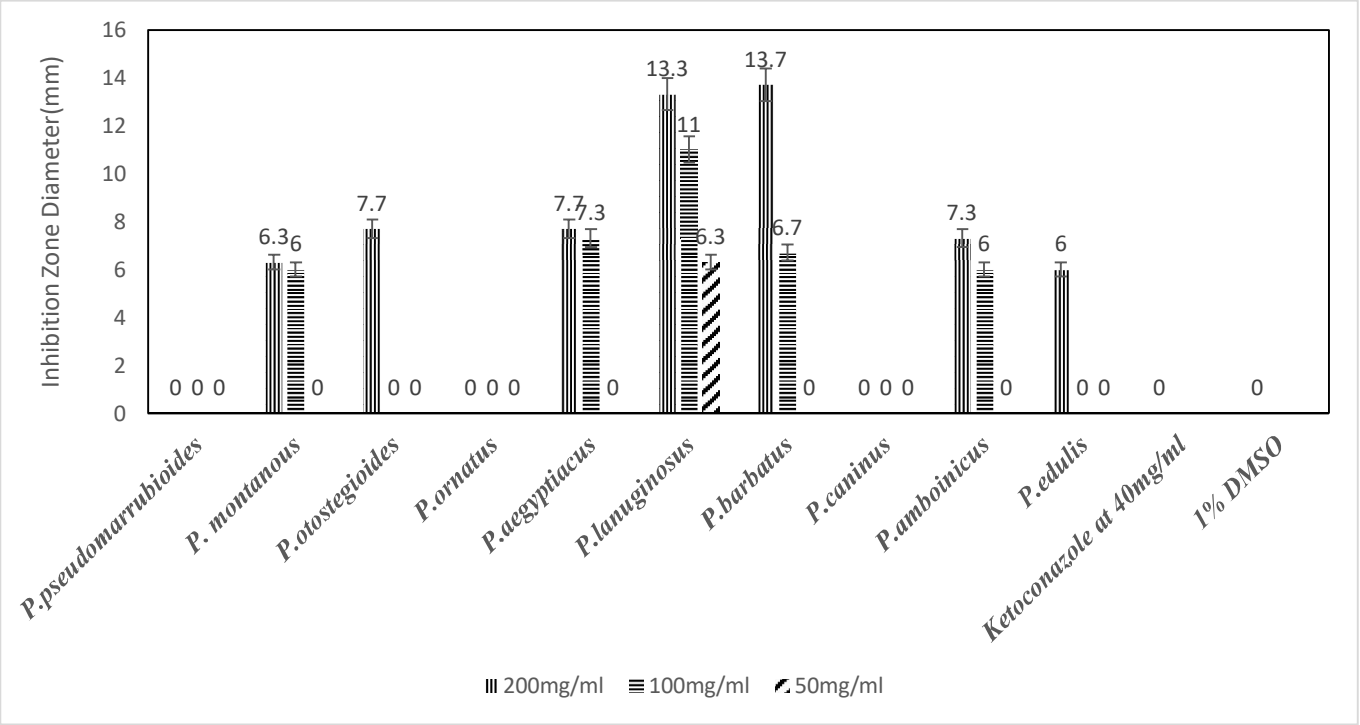


Figure 4.24: Growth inhibitions of *A. niger* by DCM-MeOH crude extracts of *Plectranthus* species at 200, 100 and 50 mg/ml

4.4.2. Analysis of antimicrobial activities of *Plectranthus* species

Antimicrobial activity of the ten *Plectranthus* species was analyzed by comparing inhibition zones of the species to determine whether there were any significant differences in the diameter of the inhibition zones among the three concentrations (200, 100 and 50 mg/ml) for each microbe among plant species using one way ANOVA. ANOVA results are presented in Table 4.5. From the analysis, the significance levels obtained after all the comparisons are < 0.001. These significance levels are less than 0.05 (Probability level/significance level used in the analysis). When the obtained significance level is less than 0.05, we conclude that the inhibition zones/ antimicrobial activity among the ten *Plectranthus* species were statistically significantly different from each other at each concentration for each microbe.

Table 4.5: Comparison of growth inhibitions of test microbes among *Plectranthus* species at 200, 100 and 50 mg/ml using ANOVA.

ANOVA						
Dependent Variable	Groups = 11(*Plectranthus* species + Control)	Sum of Squares	df	Mean Square	F	Sig.
Inhibition Zone of MRSA at 200mg/ml	Between Groups	296.667	10	29.667	61.188	**<0.001***
	Within Groups	10.667	22	.485		
	Total	307.333	32			
Inhibition Zone of MRSA at 100mg/ml	Between Groups	236.182	10	23.618	97.425	**<0.001***
	Within Groups	5.333	22	.242		
	Total	241.515	32			
Inhibition Zone of MRSA at 50 mg/ml	Between Groups	712.727	10	71.273	1176.000	**<0.001***
	Within Groups	1.333	22	.061		
	Total	714.061	32			
Inhibition Zone of *B.cereus* at 200mg/ml	Between Groups	205.636	10	20.564	56.550	**<0.001***
	Within Groups	8.000	22	.364		
	Total	213.636	32			
Inhibition Zone of *B.cereus* at 100mg/ml	Between Groups	222.242	10	22.224	73.340	**<0.001***
	Within Groups	6.667	22	.303		
	Total	228.909	32			
Inhibition Zone of *B.cereus* at 50mg/ml	Between Groups	463.636	10	46.364	306.000	**<0.001***
	Within Groups	3.333	22	.152		
	Total	466.970	32			
Inhibition Zone of *E.coli* at 200mg/ml	Between Groups	1713.394	10	171.339	1413.550	**<0.001***
	Within Groups	2.667	22	.121		
	Total	1716.061	32			
Inhibition Zone of *E.coli* at 100mg/ml	Between Groups	1756.848	10	175.685	2898.800	**<0.001***
	Within Groups	1.333	22	.061		
	Total	1758.182	32			
Inhibition Zone of *E.coli* at 50mg/ml	Between Groups	1704.545	10	170.455	.	**.**
	Within Groups	.000	22	.000		
	Total	1704.545	32			
Inhibition Zone of *C.albicans* at 200mg/ml	Between Groups	409.515	10	40.952	112.617	**<0.001***
	Within Groups	8.000	22	.364		
	Total	417.515	32			
Inhibition Zone of *C.albicans* at 100mg/ml	Between Groups	531.212	10	53.121	175.300	**<0.001***
	Within Groups	6.667	22	.303		
	Total	537.879	32			
Inhibition Zone of *C.albicans* at 50mg/ml	Between Groups	630.182	10	63.018	415.920	**<0.001***
	Within Groups	3.333	22	.152		
	Total	633.515	32			
Inhibition Zone of *A.niger* at 200mg/ml	Between Groups	787.636	10	78.764	216.600	**<0.001***
	Within Groups	8.000	22	.364		
	Total	795.636	32			
Inhibition Zone of *A.niger* at 100mg/ml	Between Groups	500.303	10	50.030	825.500	**<0.001***
	Within Groups	1.333	22	.061		
	Total	501.636	32			
Inhibition Zone of *A.niger* at 50mg/ml	Between Groups	109.394	10	10.939	361.000	**<0.001***
	Within Groups	.667	22	.030		
	Total	110.061	32			

*The inhibition means of the crude extracts are significanty different from each other when the obtained sig. level is < 0.05, from the above table, all the comparisons led to a sig. level of <0.001, implying that the means were significantly different from each other.

Antimicrobial data from the ten *Plectranthus* species was also subjected to Dunnett T test, where the inhibition zones were compared with the inhibition zones of the positive control (Amoxicillin and Ketoconazole) to determine whether any of the plants had a similar antimicrobial activity to the positive control. Details of post hoc analysis using Dunnett T test (multiple comparisons) showing the comparison of the positive controls inhibitions with the growth inhibitions of crude extracts of the study *Plectranthus* species are presented in Table 4.6.

From the analysis, the obtained significance level after comparing 200 mg/ml of *P. barbatus* and *P.lanuginosus* against MRSA with 50 mg/ml of Amoxicillin against MRSA was 0.451 and 0.439 respectively. This is > 0.05 significance level used in the analysis. Hence, it can be concluded that growth inhibition of MRSA due to 200 mg/ml of both *P. barbatus* and *P. laniginosus* was not significantly different from the growth inhibition of MRSA due to 50mg/ml of Amoxicillin. Again, it can be observed that most of the other extracts exhibited low growth inhibitions against the various bacteria tested and their low microbial growth inhibitions were significantly different (obtained p values were < 0.05) from the microbial growth inhibition of Amoxicillin (Table 4.6).

On the antifungal activity, various concentrations of the crude extracts of *Plectranthus* species have been compared with Ketoconazole as seen in Table 4.6. Comparison of 200 mg/ml of both crude extracts of *P. ornatus* and *P. barbatus* against *C.albicans* with 40mg/ml of Ketoconazole against *C. albicans* gave a siginificance level of 0.291 and 1.00 respectively. These obtained significance levels were > 0.05 significance level which set during analysis. Hence it can be concluded that, *C.albicans* growth inhibition by 200 mg/ml of *P. ornatus* and *P.barbatus* was not significantly different from *C.albicans* growth inhibition by 40 mg/ml of Ketoconazole. It can also be observed that the other extracts inhibited the growth of fungi tested although it was

significantly different from the fungal growth inhibition of Ketoconazole (obtained p values were < 0.05).

Table 4.6: Comparison between growth inhibitions of test microbes by *Plectranthus* species at 200, 100 and 50 mg/ml and the positive controls

Post hoc ANOVA: Dunnett T Test					
Dependent Variable	(I) Plant species	(J) Plant species	Mean Difference (I-J)	Std. Error	Sig.
Inhibition Zone of MRSA at 200mg/ml	***P.lanuginosus***	**Amoxicillin at 50mg/ml**	**1.00000**	**0.56854**	**0.439**
	P.barbatus	**Amoxicillin at 50mg/ml**	**-1.00000**	**0.56854**	**0.451**
Inhibition Zone of MRSA at 100mg/ml	*P.lanuginosus*	Amoxicillin at 50mg/ml	-1.66667*	0.40202	0.003
	P.barbatus	Amoxicillin at 50mg/ml	-3.66667*	0.40202	0.000
Inhibition Zone of MRSA at 50 mg/ml	*P.lanuginosus*	Amoxicillin at 50mg/ml	-8.00000*	0.20101	0.000
	P.barbatus	Amoxicillin at 50mg/ml	-7.66667*	0.20101	0.000
Inhibition Zone of *B.cereus* at 200mg/ml	*P.osteogioides*	Amoxicillin at 50mg/ml	-2.66667*	0.49237	0.000
	P.lanuginosus	Amoxicillin at 50mg/ml	-2.66667*	0.49237	0.000
	P.barbatus	Amoxicillin at 50mg/ml	-2.00000*	0.49237	0.004
Inhibition Zone of *B.cereus* at 100mg/ml	*P.osteogioides*	Amoxicillin at 50mg/ml	-5.33333*	0.44947	0.000
	P.lanuginosus	Amoxicillin at 50mg/ml	-4.33333*	0.44947	0.000
	P.barbatus	Amoxicillin at 50mg/ml	-6.33333*	0.44947	0.000
Inhibition Zone of *B.cereus* at 50mg/ml	*P.osteogioides*	Amoxicillin at 50mg/ml	-8.33333*	0.31782	0.000
	P.lanuginosus	Amoxicillin at 50mg/ml	-8.33333*	0.31782	0.000
	P.barbatus	Amoxicillin at 50mg/ml	-8.33333*	0.31782	0.000
Inhibition Zone of *E.coli* at 200mg/ml	*P.barbatus*	Amoxicillin at 50mg/ml	-15.00000*	0.28427	0.000
	P.edulis	Amoxicillin at 50mg/ml	-15.66667*	0.28427	0.000
Inhibition Zone of *E.coli* at 100mg/ml	*P.barbatus*	Amoxicillin at 50mg/ml	-17.33333*	0.20101	0.000
	P.edulis	Amoxicillin at 50mg/ml	-18.66667*	0.20101	0.000
Inhibition Zone of *C.albicans* at 200mg/ml	***P.ornatus***	**Ketakonazol at 40 mg/ml**	**-1.00000**	**0.49237**	**0.290**
	P.barbatus	**Ketakonazol at 40 mg/ml**	**0.00000**	**0.49237**	**1.000**
Inhibition Zone of *C.albicans* at 100mg/ml	*P.ornatus*	Ketakonazol at 40 mg/ml	-5.33333*	0.44947	0.000
	P.barbatus	Ketakonazol at 40 mg/ml	-5.00000*	0.44947	0.000
Inhibition Zone of *C.albicans* at 50mg/ml	*P.ornatus*	Ketakonazol at 40 mg/ml	-7.66667*	0.31782	0.000
	P.barbatus	Ketakonazol at 40 mg/ml	-7.33333*	0.31782	0.000
Inhibition Zone of *A.niger* at 200mg/ml	*P.lanuginosus*	Ketakonazol at 40 mg/ml	13.33333*	0.49237	0.000
	P.barbatus	Ketakonazol at 40 mg/ml	13.66667*	0.49237	0.000
Inhibition Zone of *A.niger* at 100mg/ml	*P.lanuginosus*	Ketakonazol at 40 mg/ml	11.00000*	0.20101	0.000
	P.barbatus	Ketakonazol at 40 mg/ml	6.66667*	0.20101	0.000
Inhibition Zone of *A.niger* at 50mg/ml	*P.lanuginosus*	Ketakonazol at 40 mg/ml	6.33333*	0.14213	0.000
	P.barbatus	Ketakonazol at 40 mg/ml	0.00000	0.14213	0.000
*. The inhibition means of the crude extracts are significanty different from the positive controls means when the obtained sig. level is < 0.05. When the obtained sig. level is ≥ 0.05, the inhibition means are not significantly different from the positive control means.					
a. Dunnett t-tests treat one group as a control, and compare all other groups against it.					

4.4.3. Minimum inhibitory concentration (MIC) values of total extracts from studied *Plectranthus* species

Dichloromethane/methanol [DCM: MeOH (1:1)] crude organic extract from each of the 10 species was subjected to antimicrobial activity through broth dilution and the results are presented in Table 4.7. From the preliminary antimicrobial activity screening of DCM: MEoH crude extracts from the ten species, it was observed that all the species inhibited the growth of MRSA and *B.cereus*. Observed MIC values for *P.pseudomarrubioides, P.montanous, P.otostegioides, P.ornatus, P.aegyptiacus, P.lanuginosus, P.barbatus, P.amboinicus, P.edulis* against MRSA were 100 mg/ml, 75 mg/ml, 100mg/ml, 50 mg/ml, 40 mg/ml, 40 mg/ml, 25 mg/ml, 50mg/ml, 100mg/ml and 75 mg/ml respectively. MIC values for *P.pseudomarrubioides, P.montanous, P.otostegioides, P.ornatus, P.aegyptiacus, P.lanuginosus, P.barbatus, P.amboinicus, P. edulis* against *B.cereus* were 100 mg/ml, 50 mg/ml, 40mg/ml, 50 mg/ml, 100 mg/ml, 50 mg/ml, 40 mg/ml, 50mg/ml, 50mg/ml and 40 mg/ml respectively. For *E.coli*, the MIC values were above 200 mg/ml except for *P.barbatus* and *P.edulis* which both had a MIC value of 100 mg/ml against *E.coli*.

On the other hand, MIC values for *P.pseudomarrubioides, P.montanous, P.otostegioides, P.ornatus, P.aegyptiacus, P.lanuginosus, P.barbatus, P.amboinicus, P.edulis* against *C.albicans* were 100 mg/ml, 50 mg/ml, 50mg/ml, 50 mg/ml, 50 mg/ml, 150 mg/ml, 50 mg/ml, <200 mg/ml, 75mg/ml and 150 mg/ml respectively. Lastly for *A.niger*, *P.pseudomarrubioides*, *P.ornatus* and *P.caninus* had a MIC values of <200 mg/ml while MIC values for *P.montanous, P.otostegioides, P.aegyptiacus, P.lanuginosus, P.barbatus, P.amboinicus, P.edulis* against *A.niger* were 200 mg/ml, 150 mg/ml, 100 mg/ml, 150 mg/ml, 100 mg/ml, 200mg/ml and 200 mg/ml respectively (Table 4.7). Broth dilution results indicate that, *P.barbatus* had the lowest MIC values against the test microbes compared to the other *Plectranthus* species.

Table 4.7: Minimum inhibitory concentration of *Plectranthus* species

Plant species	**Minimum Inhibitory Concentrations (mg/ml)**				
	MRSA	***B.cereus***	***E.coli***	***C.albicans***	***A.niger***
P.pseudomarrubioides	100	100	No inhibition/>200	100	No inhibition/>200
P.montanous	75	50	No inhibition/>200	50	200
P.ostegioides	100	40	No inhibition/>200	50	150
P.ornatus	50	50	No inhibition/>200	50	No inhibition/>200
P.aegyptiacus	40	100	No inhibition/>200	50	100
P.lanuginosus	40	50	No inhibition/>200	150	50
P.barbatus	25	40	100	50	100
P.caninus	50	50	No inhibition/>200	No inhibition/>200	No inhibition/>200
P.amboinicus	100	50	No inhibition/>200	75	200
P.edulis	75	40	100	150	200

4.5. Antimicrobial activity of fractionated extracts of *P.barbatus*

4.5.1. Disc diffusion results

Crude ethanol extract of *P. barbatus* was sequentially partitioned to give five fractions/partitions. These were Petroleum ether, n-hexane, DCM, chloroform and EtoAC. All the fractions were investigated for their antimicrobial activity using disc diffusion technique. The results are represented in the following figures.

Only hexane and DCM fractions of *P.barbatus* inhibited the growth of MRSA. Average growth inhibition diameter for 200 mg/ml of hexane fraction was 7.7 mm while average growth inhibition diameters for 200 mg/ml, 100 mg/ml and 50 mg/ml of the DCM fraction were 8.3 mm, 7.7 mm and 6 mm respectively. Average growth inhibition by Amoxicillin (50 mg/ml) was 14 mm while 1% DMSO did not inhibit the growth of MRSA (Figure 4.25).

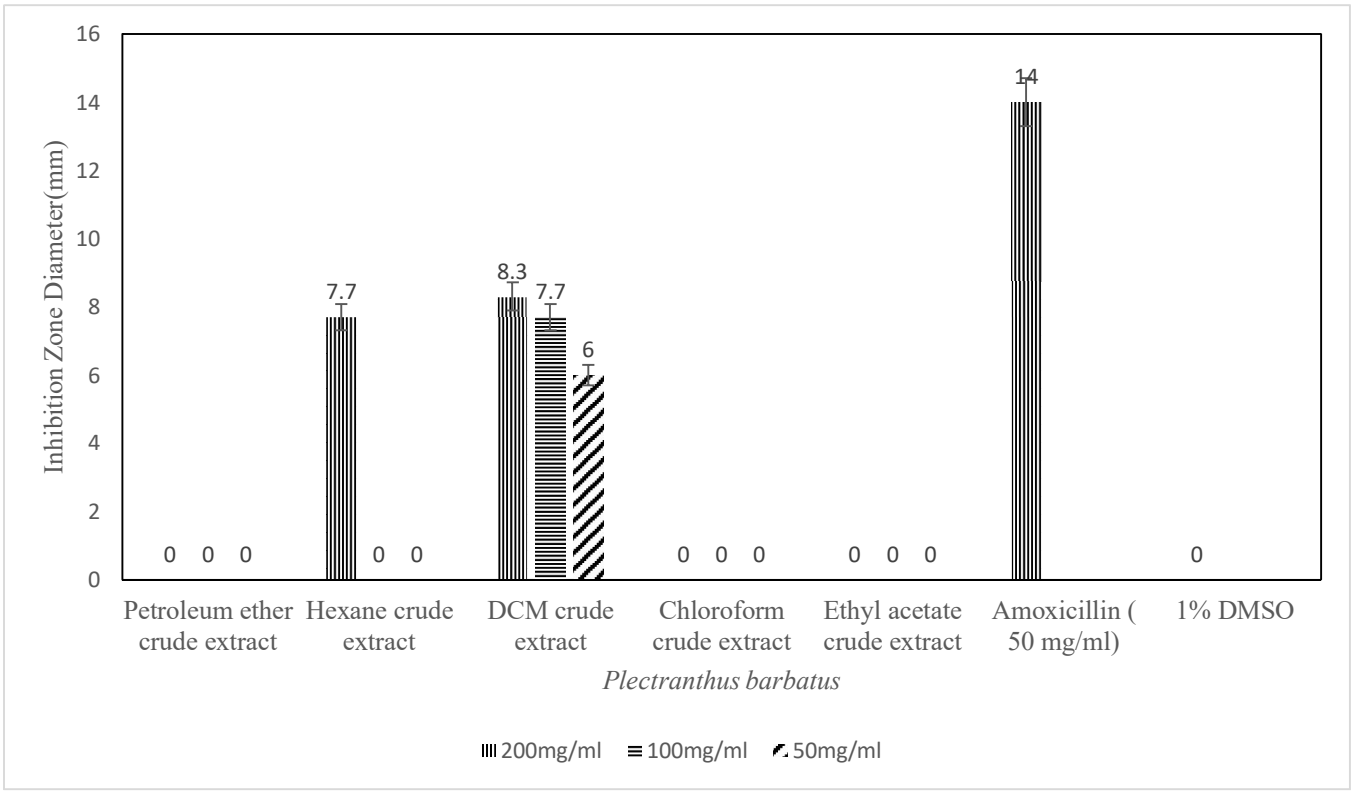


Figure 4.25: Growth inhibitions of MRSA by *P.barbatus* fractionated crude extracts

All the generated fractions of *P.barbatus* inhibited the multiplication of *B.cereus* in a dose dependent manner except the hexane fraction. 200 mg/ml and 100 mg/ml of petroleum ether fraction exhibited growth inhibition diameters of 9.3 mm and 8 mm respectively, 200 mg/ml of the DCM fraction exhibited a growth inhibition diameter of 9 mm. Average growth inhibition diameters for the 200 mg/ml and 100 mg/ml of the chloroform fraction were 12 mm and 8.3 mm respectively while growth inhibition diameters for both 200 mg/ml and 100 mg/ml of the ethyl acetate fraction were 11.3 mm and 9.3 mm respectively. Average inhibition zone for the positive control [Amoxicillin (50 mg/ml)] was 15 mm while the negative control (1% DMSO) did not inhibit growth of *B.cereus* (Figure 4.26).

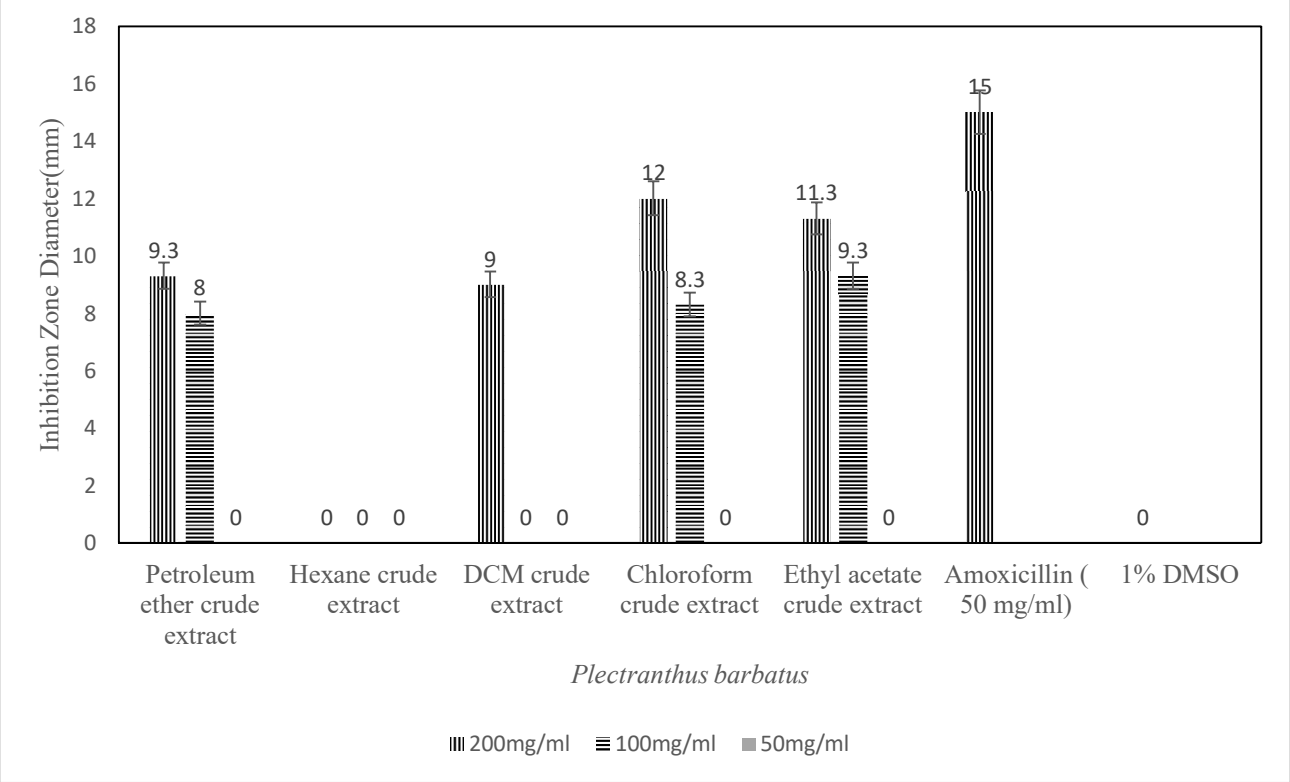


Figure 4.26: Growth inhibitions of *B.cereus* by *P.barbatus* fractionated crude extracts

All generated fractions inhibited growth of *E.coli* in a dose dependent manner. Growth inhibition diameters of both 200 mg/ml and 100 mg/ml of the petroleum ether fraction were 10.3 mm and 7.7 mm respectively. Growth inhibition diameters of 200 mg/ml and 100 mg/ml of the hexane fraction were 8.3 mm and 7.7 mm. 200 mg/ml, 100 mg/ml and 50 mg/ml of the DCM fraction exhibited growth inhibition diameters of 13.3 mm, 10.7 mm and 10 mm respectively while 200 mg/ml, 100 mg/ml and 50 mg/ml of the chloroform fraction exhibited growth inhibition diameters of 11 mm, 10.3 mm and 8.3 mm respectively. Both 200 mg/ml and 100 mg/ml of the ethyl acetate fraction exhibited a growth inhibition diameter of 8.7 mm and 8 mm respectively while the for the controls, the average inhibition zone for the positive control [Amoxicillin (50 mg/ml)] was 25 mm while the negative control (1% DMSO) did not inhibit growth of *E.coli* (Figure 4.27).

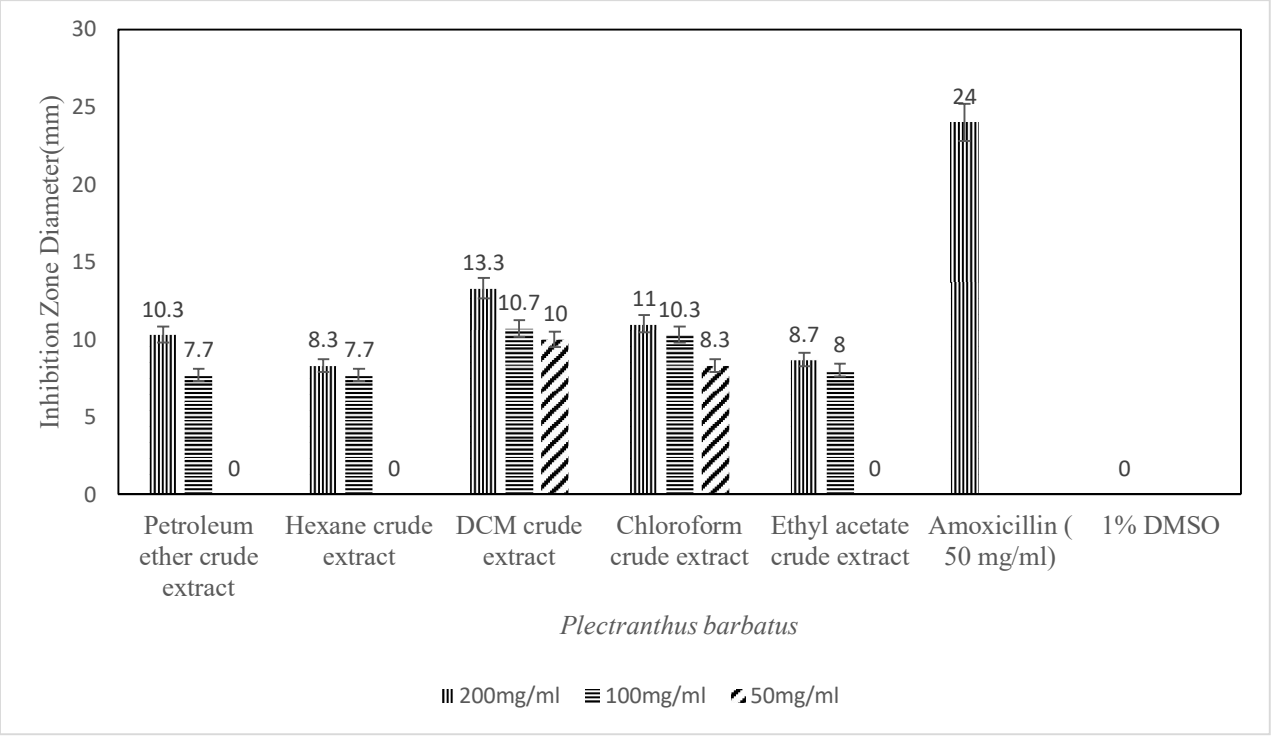


Figure 4.27: Growth inhibitions of *E.coli* by *P. barbatus* fractionated extracts

All generated fractions of *P.barbatus* inhibited growth of *C.albicans* in a dose dependent manner. Average growth inhibition diameters of both 200 mg/ml and 100 mg/ml of the petroleum ether fraction were 10 mm and 9 mm respectively. Growth inhibition diameters of 200 mg/ml and 100 mg/ml of the hexane fraction were 8 mm and 6.7 mm. 200 mg/ml, 100 mg/ml and 50 mg/ml of the DCM fraction exhibited growth inhibition diameters oof 11.7 mm, 10.7 mm and 8.3 mm respectively while 200 mg/ml, 100 mg/ml and 50 mg/ml of the chloroform fraction exhibited growth inhibition diameters of 10 mm, 9.3 mm and 7.7 mm respectively. Both 200 mg/ml and 100 mg/ml of the ethyl acetetae fraction exhibited a growth inhibition diameter of 8.3 mm and 6.3 mm respectively while the for the controls, the average growth inhibition diameter of ketoconazole (40 mg/ml) was 14 mm while 1% DMSO did not inhibit the growth of *C. albicans* (Figure 4.28).

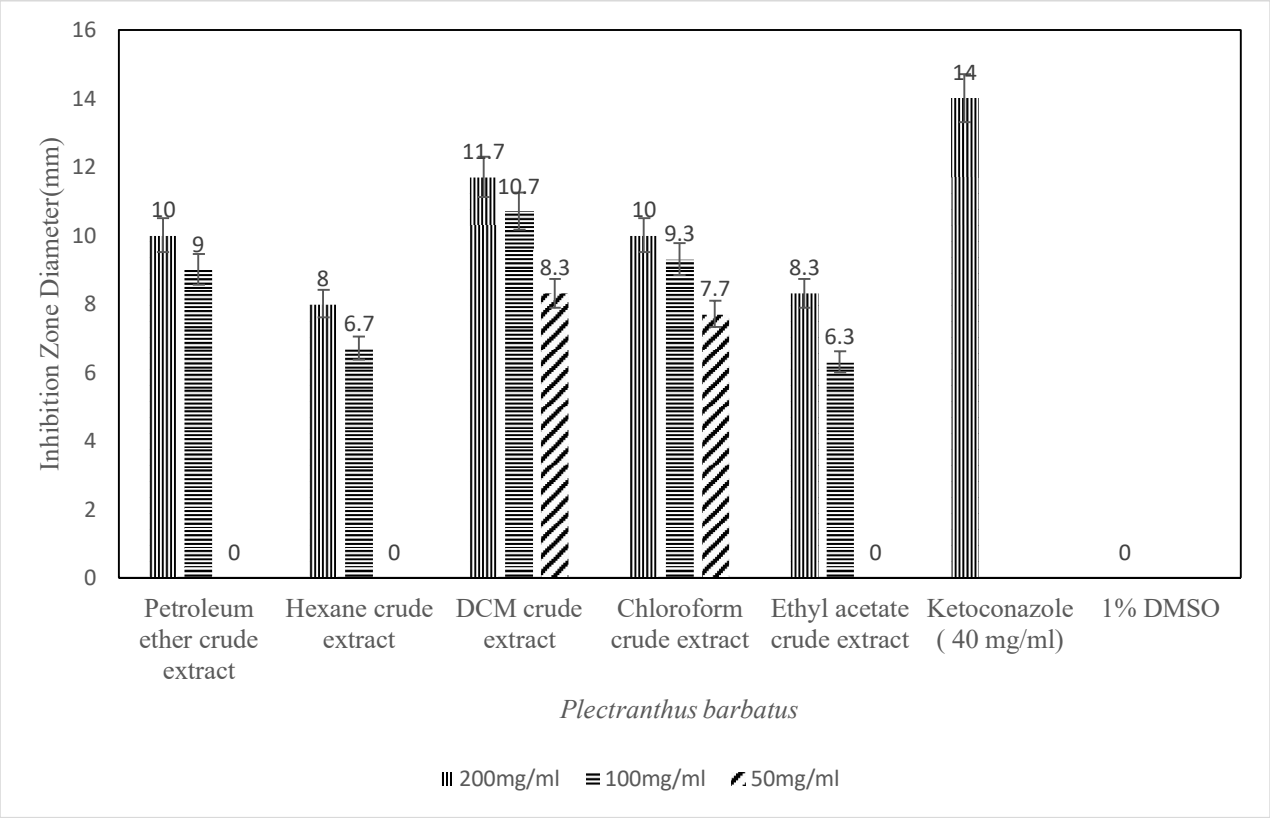


Figure 4.28: Growth inhibitions of *C.albicans* by *P.barbatus* fractionated crude extracts

Only the hexane and DCM fractions of *P.barbatus* inhibited the multiplication of *A.niger*. Average growth inhibition diameters of the 200 mg/ml of the hexane fraction, 200 mg/ml and 100 mg/ml of the DCM fraction were 8.7 mm, 9.7 mm and 6.7 mm respectively. The other fractions of *P.barbatus* together with the control did not inhibit the growth of *A.niger* (Figure 4.29).

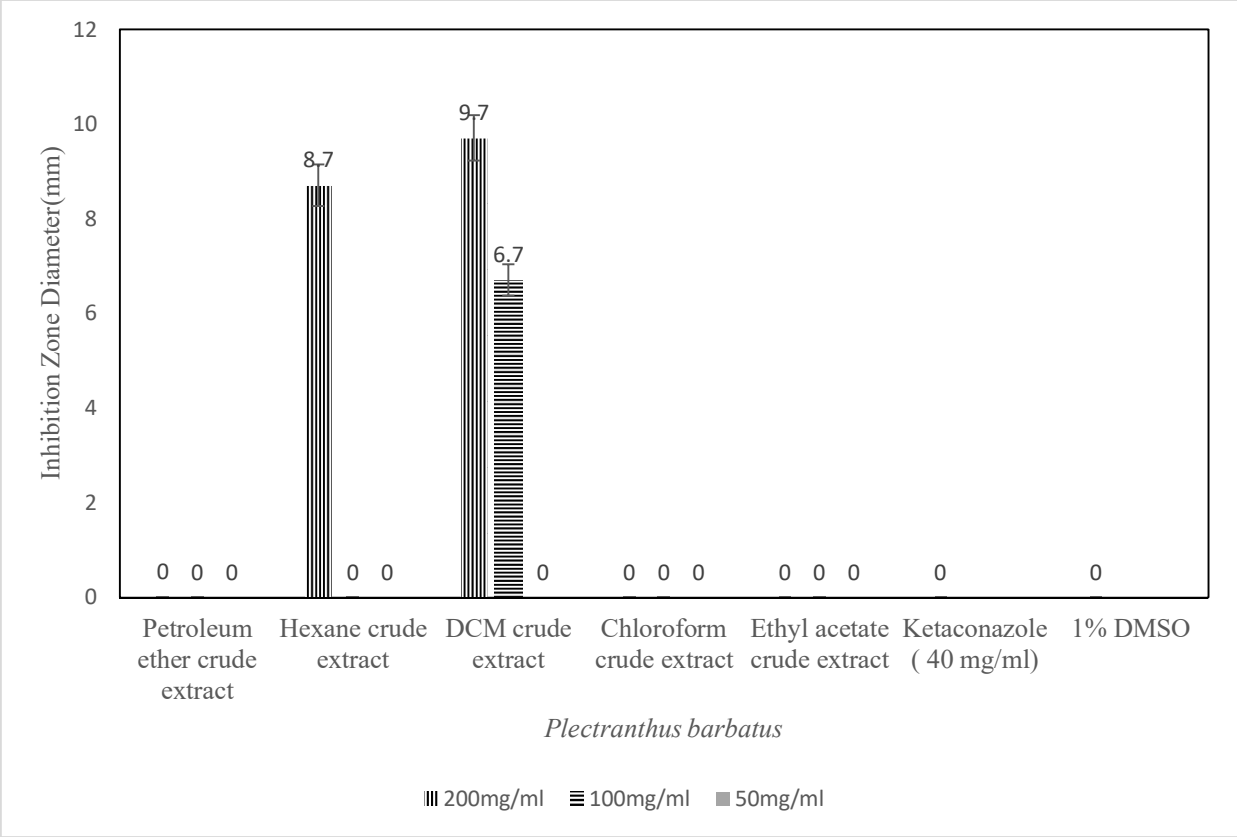


Figure 4.29: Growth inhibitions of *A.niger* by *P.barbatus* fractionated crude extracts

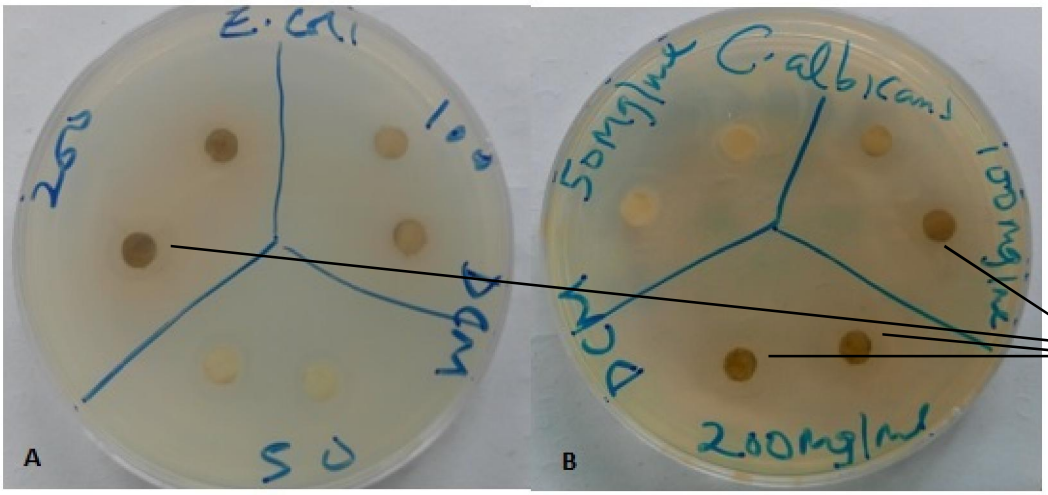


Figure 4.30: Inhibition zones of the DCM extracts of *P.barbatus*

A: Growth inhibition of *E.coli* by *P.barbatus* DCM extracts, **B:** Growth inhibition of *C.albicans* by *P.barbatus* DCM extracts.

4.5.2. Analysis of antimicrobial activities of *P. barbatus* fractionated crude extracts

Growth inhibitions of partitions of *P. barbatus* was analyzed by comparing whether there were any significant differences in the diameter of the inhibition zones among the three concentrations (200, 100 and 50mg/ml) for each microbe among the *P.barbatus* partitions using one way ANOVA. The results of the analysis are presented in Table 4.8. From the analysis, all the comparisons gave a significance level of < 0.001. This obtained significance leves of <0.001 were less than the 0.05 significance level used in analaysis. This implies growth inhibitions among the partitions of *P.barbatus* were statistically significantly different from each other at each concentration for each microbe.

Table 4.8: Comparison of growth inhibitions of test microbes among *P.barbatus* fractionated crude extracts at 200, 100 and 50 mg/ml

ANOVA						
Dependent Variable	Groups = 6 (partions + control)	Sum of Squares	df	Mean Square	F	Sig.
Inhibition Zone of MRSA at 200mg/ml	Between Groups	522.667	5	104.533	940.800	**<0.001***
	Within Groups	1.333	12	.111		
	Total	524.000	17			
Inhibition Zone of MRSA at 100mg/ml	Between Groups	529.611	5	105.922	1906.600	**<0.001***
	Within Groups	.667	12	.056		
	Total	530.278	17			
Inhibition Zone of MRSA at 50 mg/ml	Between Groups	562.500	5	112.500	.	**.**
	Within Groups	.000	12	.000		
	Total	562.500	17			
Inhibition Zone of *B.cereus* at 200mg/ml	Between Groups	360.278	5	72.056	117.909	**<0.001***
	Within Groups	7.333	12	.611		
	Total	367.611	17			
Inhibition Zone of *B.cereus* at 100mg/ml	Between Groups	509.778	5	101.956	367.040	**<0.001***
	Within Groups	3.333	12	.278		
	Total	513.111	17			
Inhibition Zone of *B.cereus* at 50mg/ml	Between Groups	562.500	5	112.500	.	**.**
	Within Groups	.000	12	.000		
	Total	562.500	17			
Inhibition Zone of *E.coli* at 200mg/ml	Between Groups	515.611	5	103.122	265.171	**<0.001***
	Within Groups	4.667	12	.389		
	Total	520.278	17			
Inhibition Zone of *E.coli* at 100mg/ml	Between Groups	677.778	5	135.556	348.571	**<0.001***
	Within Groups	4.667	12	.389		
	Total	682.444	17			
Inhibition Zone of *E.coli* at 50mg/ml	Between Groups	1444.444	5	288.889	5200.000	**<0.001***
	Within Groups	.667	12	.056		
	Total	1445.111	17			
Inhibition Zone of *C.albicans* at 200mg/ml	Between Groups	55.167	5	11.033	24.825	**<0.001***
	Within Groups	5.333	12	.444		
	Total	60.500	17			
Inhibition Zone of *C.albicans* at 100mg/ml	Between Groups	119.333	5	23.867	61.371	**<0.001***
	Within Groups	4.667	12	.389		
	Total	124.000	17			
Inhibition Zone of *C.albicans* at 50mg/ml	Between Groups	522.667	5	104.533	940.800	**<0.001***
	Within Groups	1.333	12	.111		
	Total	524.000	17			
Inhibition Zone of *A.niger* at 200mg/ml	Between Groups	337.611	5	67.522	243.080	**<0.001***
	Within Groups	3.333	12	.278		
	Total	340.944	17			
Inhibition Zone of *A.niger* at 100mg/ml	Between Groups	111.111	5	22.222	400.000	**<0.001***
	Within Groups	.667	12	.056		
	Total	111.778	17			
Inhibition Zone of *A.niger* at 50mg/ml	Between Groups	.000	5	.000	.	**.**
	Within Groups	.000	12	.000		
	Total	.000	17			

*. The inhibition means of the fractions are significanty different from each other when the obtained sig. level is < 0.05, from the above table, all the comparisons led to a sig. level of <0.001, implying that the inhibition means were significantly different from each other

Since there was a control group, the zone of inhibitions of *P.barbatus* partitions were compared with the positive control inhibitions to determine whether they were significantly different or not from the positive controls using Dunnett T test (post hoc analysis test). The results of the analysis are presented in Table 4.9 overleaf. From the Dunnet T test, comparison of inhibitions of various concentrations of each partition with that of the respective positive control gave a significance values of <0.001 except in the comparison of antifungal activity of 200 mg/ml of the DCM partition against *C.albicans* with ketaconzole activity which gave a significance level of 0.109 (Table 4.9). This value of 0.109 is > 0.05; the significance level used in the analysis. This implies all the partitions of *P.barbatus* inhibited the growth of microbes tested and their inhibitions were significantly different from the positive control; amoxicillin or ketoconazole (obtained $p = 0.000 < 0.05$) inhibition except DCM partition which had significantly similar anticandida activity to 40 mg/ml of ketoconazole (obtained $p = 0.109 > 0.05$)

In addition, from the column on Mean Difference (I-J) in Table 4.9, it is possible to tell which mean was close to that of the positive control. Hence by looking at the DCM and chloroform crude extracts of *P.barbatus* in all the comparisons, we find that their mean was close to the positive control mean (indicated by a small mean difference) implying they had higher microbial growth inhibitions compared to the other partitions although statistically significantly different from the microbial growth inhibition of the positive controls.

Table 4.9: Comparison between growth inhibitions of test microbes by *P.barbatus* fractionated crude extracts at 200, 100 and 50 mg/ml and positive controls

Post Hoc ANOVA: Dunnett T Test					
Dependent Variable	(I) *P.* barbatus partitions	(J) Positive Control	Mean Difference (I-J)	Std. Error	Sig.
	Petroleum ether crude extract	Amoxicillin (50 mg/ml)	-14.00000*	0.27217	<0.001
	Hexane crude extract	**Amoxicillin (50 mg/ml)**	**-6.33333***	0.27217	<0.001
	DCM crude extract	**Amoxicillin (50 mg/ml)**	**-5.66667***	0.27217	<0.001
	Chloroform crude extract	Amoxicillin (50 mg/ml)	-14.00000*	0.27217	<0.001
	Ethyl acetate crude extract	Amoxicillin (50 mg/ml)	-14.00000*	0.27217	<0.001
Inhibition Zone of MRSA at 100mg/ml	Petroleum ether crude extract	Amoxicillin (50 mg/ml)	-14.00000*	0.19245	<0.001
	Hexane crude extract	Amoxicillin (50 mg/ml)	-14.00000*	0.19245	<0.001
	DCM crude extract	**Amoxicillin (50 mg/ml)**	**-6.33333***	0.19245	<0.001
	Chloroform crude extract	Amoxicillin (50 mg/ml)	-14.00000*	0.19245	<0.001
	Ethyl acetate crude extract	Amoxicillin (50 mg/ml)	-14.00000*	0.19245	<0.001
Inhibition Zone of *B.cereus* at 200mg/ml	Petroleum ether crude extract	Amoxicillin (50 mg/ml)	-4.66667*	0.63828	<0.001
	Hexane crude extract	Amoxicillin (50 mg/ml)	-14.00000*	0.63828	<0.001
	DCM crude extract	**Amoxicillin (50 mg/ml)**	**-5.00000***	0.63828	<0.001
	Chloroform crude extract	**Amoxicillin (50 mg/ml)**	**-2.00000***	0.63828	0.033
	Ethyl acetate crude extract	**Amoxicillin (50 mg/ml)**	**-2.66667***	0.63828	0.005
Inhibition Zone of *B.cereus* at 100mg/ml	Petroleum ether crude extract	Amoxicillin (50 mg/ml)	-7.00000*	0.43033	<0.001
	Hexane crude extract	Amoxicillin (50 mg/ml)	-15.00000*	0.43033	<0.001
	DCM crude extract	Amoxicillin (50 mg/ml)	-15.00000*	0.43033	<0.001
	Chloroform crude extract	Amoxicillin (50 mg/ml)	-6.66667*	0.43033	<0.001
	Ethyl acetate crude extract	**Amoxicillin (50 mg/ml)**	**-5.66667***	0.43033	<0.001
Inhibition Zone of *E.coli* at 200mg/ml, 100 mg/ml & 50 mg/ml	Petroleum ether crude extract	Amoxicillin (50 mg/ml)	-13.66667*	0.50918	<0.001
	Hexane crude extract	Amoxicillin (50 mg/ml)	-15.66667*	0.50918	<0.001
	DCM crude extract	Amoxicillin (50 mg/ml)	-10.66667*	0.50918	<0.001
	Chloroform crude extract	Amoxicillin (50 mg/ml)	-13.00000*	0.50918	<0.001
	Ethyl acetate crude extract	Amoxicillin (50 mg/ml)	-15.33333*	0.50918	<0.001
Inhibition Zone of *C.albicans* at 200mg/ml	**Petroleum ether crude extract**	**Ketoconazole (40 mg/ml)**	**-3.00000***	0.54433	0.001
	Hexane crude extract	Ketoconazole (40 mg/ml)	-5.00000*	0.54433	<0.001
	DCM crude extract	**Ketoconazole (40 mg/ml)**	**-1.33333**	**0.54433**	**0.109**
	Chloroform crude extract	**Ketoconazole (40 mg/ml)**	**-3.00000***	0.54433	<0.001
	Ethyl acetate crude extract	**Ketoconazole (40 mg/ml)**	**-4.66667***	0.54433	<0.001
Inhibition Zone of *C.albicans* at 100mg/ml	Petroleum ether crude extract	Ketoconazole (40 mg/ml)	-5.00000*	0.50918	<0.001
	Hexane crude extract	Ketoconazole (40 mg/ml)	-7.33333*	0.50918	<0.001
	DCM crude extract	**Ketoconazole (40 mg/ml)**	**-3.33333***	0.50918	<0.001
	Chloroform crude extract	**Ketoconazole (40 mg/ml)**	**-4.66667***	0.50918	<0.001
	Ethyl acetate crude extract	Ketoconazole (40 mg/ml)	-7.66667*	0.50918	<0.001
Inhibition Zone of *C.albicans* at 50mg/ml	Petroleum ether crude extract	Ketoconazole (40 mg/ml)	-14.00000*	0.27217	<0.001
	Hexane crude extract	Ketoconazole (40 mg/ml)	-14.00000*	0.27217	<0.001
	DCM crude extract	**Ketoconazole (40 mg/ml)**	**-5.66667***	0.27217	<0.001
	Chloroform crude extract	**Ketoconazole (40 mg/ml)**	**-6.33333***	0.27217	<0.001
	Ethyl acetate crude extract	Ketoconazole (40 mg/ml)	-14.00000*	0.27217	<0.001
Inhibition Zone of *A.niger* at 100 and 200mg/ml	Petroleum ether crude extract	Ketoconazole (40 mg/ml)	0.00000	0.43033	<0.001
	Hexane crude extract	Ketoconazole (40 mg/ml)	8.66667*	0.43033	<0.001
	DCM crude extract	Ketoconazole (40 mg/ml)	9.66667*	0.43033	<0.001
	Chloroform crude extract	Ketoconazole (40 mg/ml)	0.00000	0.43033	<0.001
	Ethyl acetate crude extract	Ketoconazole (40 mg/ml)	0.00000	0.43033	<0.001
*. The inhibition means of the fractions are significanty different from the positive controls means when the obtained sig. level is < 0.05. When the obtained sig. level is ≥ 0.05, the inhibition means are not significantly different from the positive control means.					
a. Dunnett t-tests treat one group as a control, and compare all other groups against it.					

4.5.3. Minimum Inhibitory Concentrations of *P. barbatus* fractionated crude extracts

Fractionated crude extracts of *P. barbatus* were subjected to antimicrobial activity through broth dilution and the results are represented in Table 4.10. From the results, DCM (MIC value = 40 mg/ml) and hexane (MIC value = 150mg/ml) leaf crude extracts of *P.barbatus* were found to have activity against MRSA while petroleum ether, chloroform and ethyl acetate crude extracts did not inhibit the growth of MRSA. Secondly, minimum inhibition values of the leaf different crude extracts of *P.barbatus* against *B. cereus* were as follows; petroleum ether (MIC value = 75mg/ml), DCM (MIC value = 150 mg/ml), chloroform (MIC value = 75 mg/ml), EtoAC (MIC value = 60 mg/ml) and n-hexane (> 200 mg/ml). All the crude extracts of *P.barbatus* were tested against *E.coli* and *C.albicans*were found to inhibit the growth of *E.coli* and *C.albicans.* The MIC values for petroleum ether, n-hexane, DCM, chloroform and EtoAc extracts against *E.coli* were 75 mg/ml, 75 mg/ml, 25 mg/ml, 25 mg/ml and 75 mg/ml respectively while MIC values for *C.albicans* for petroleum ether, n-hexane, DCM, Chloroform and EtoAc extracts were 60 mg/ml, 75 mg/ml, 25 mg/ml, 25 mg/ml and 75 mg/ml respectively. Lastly, hexane and DCM crude extracts of *P.barbatus* had MIC values 150 mg/ml and 75 mg/ml respectively against *A.niger* while petroleum ether, chloroform and EtoAC had a MIC values of > 200 mg/ml respectively against *A.niger* (Table 4.10). The results indicate that DCM partition of *P.barbatus* had the lowest MIC values against all the microbes tested compared to the other four *P.barbatus* partitions.

Table 4.10: Minimum inhibitory concentrations of *P.barbatus* fractionated crude extracts

***P. barbatus* partition**	**Minimum Inhibitory Concentrations (mg/ml)**				
	MRSA	***B.cereus***	***E.coli***	***C.albicans***	***A.niger***
Petroleum ether	No inhibition/>200	75	75	60	No inhibition/>200
n-hexane	150	No inhibition/>200	75	75	150
DCM	40	150	25	25	75
Chloroform	No inhibition/>200	75	25	25	No inhibition/>200
Ethylacetate	No inhibition/>200	60	75	75	No inhibition/>200

4.6.Thin Layer Chromatography (TLC) profiles of *P. barbatus* isolated compounds

After subjecting the DCM fraction of *P.barbatus* to column chromatography, fractions with several compounds were obtained as ascertained from TLC profiles. Fractions with similar TLC profiles were combined and some of the fractions were subjected to further column chromatography and it was possible to identify two compounds of sizeable amount; compound A (1.1g) and Compound B (2.3g). Each of the compounds displayed a single spot on a TLC plate after being immersed in iodine chamber and after observing them under UV light. Single spots on TLC plate indicates that the compound is a single compound while several spots on a TLC plate indicates that the sample has more than one compound and further isolation is needed to get pure compounds. The TLC Rf values for compound A and B in 100% DCM were 0.500 and 0.375, respectively.

4.7. Determination of the presence of isolated compounds A and B in other *Plectranthus* species

Presence of the isolated compounds A and B in the other 9 *Plectranthus* species were determined by TLC and UV spectroscopy. Similar spot with the same Retention factors (Rf) values appearing in other species indicates the presence of the compounds in other species as well. The presence of the two compounds A and B in some of the other nine (9) *Plectranthus* species is represented in Figures 4.31 and 4.32.

Apart from being present in *P. barbatus*, Compound A was also present in *P. otostegioides*, *P. ornatus* and *P. caninus* (Figure 4.31).

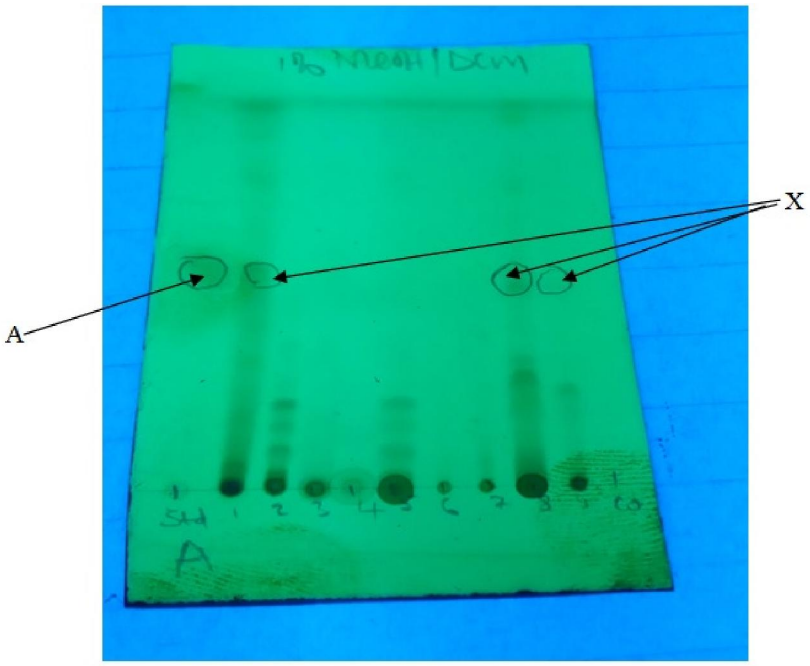


Figure 4.31: Detection of compound A in other *Plectranthus* species

Key: *1=P.otostegioides, 2=P.amboinicus, 3=P.lanuginosus, 4=P.edulis, 5=P.montanous, 6=P.pseudomarrubioides, 7=P. aegyptiacus, 8=P.ornatus, 9=P.caninus.* **A:** Compound A in *P.barbatus,* **X**: Compound A in other *Plectranthus* species.

Apart from being present in *P. barbatus*, compound B is also present in *P. amboinicus, P. montanous, P. ornatus* and *P. caninus* (Figure 4.32).

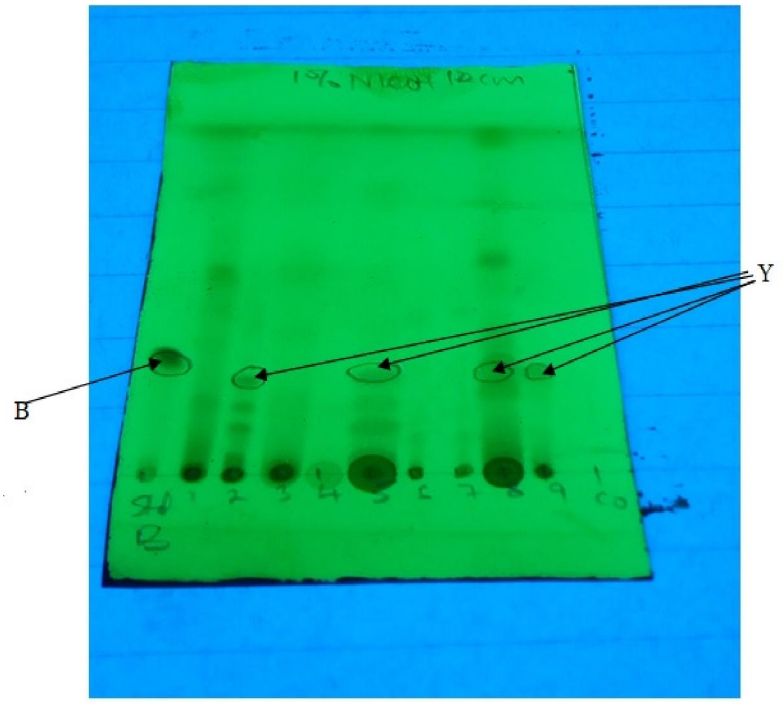


Figure 4.32: Detection of compound B in other *Plectranthus* species

Key: *1=P.otostegioides, 2=P.amboinicus, 3=P.lanuginosus, 4=P.edulis, 5=P.montanous, 6=P.pseudomarrubioides, 7=P. aegyptiacus, 8= P.ornatus, 9=P.caninus,* **B**: Compound B in *P. barbatus*, **Y**: Compound B in other *Plectranthus* species

4.8. Antimicrobial activity of the isolated compounds A and B

Antimicrobial activity of these compounds was determined by broth dilution technique to determine MICs of the compounds against *MRSA, B.cereus, E.coli, C.albicans* and *A.niger.* The lowest concentration with no visible bacterial or fungal growth was regarded as the MIC for the respective microbe. The results are summarised in Table 4.11.

For compound A, MIC values were 40 mg/ml, 40 mg/ml, 50 mg/ml, 50 mg/ml and 40 mg/ml for MRSA, *B.cereus, E.coli, C.albicans, A. niger* respectively. For compound B, the MIC values were 20 mg/ml, 50 mg/ml, 20 mg/ml, 40 mg/ml and 50 mg/ml for MRSA, *B.cereus, E.coli, C.albicans, A. niger* respectively. The lowest MIC values of 20 mg/ml were observed when compound B was evaluated against MRSA and *E. coli.* (Table 4.11). As earlier mentioned, antimicrobial activity for compounds, the antimicrobial activity is significant when MIC<10 µg/mL, moderate when 10<MIC<100 µg/mL and negligible or low when MIC>100 µg/mL (Kuete, 2010). Observed MICs values from both compounds A and B against the five microbes under study were above 100 µg/ml. Hence the two compounds can be said to have low antimicrobial activity against the test microbes.

Table 4.11: Determination of MICs of Compound A & B against the test mirobes

	Antimicrobial activity of isolated compound A					MIC
Microbes	50mg/ml	40mg/ml	30 mg/ml	20mg/ml	10mg/ml	mg/ml
MRSA	- ve	- ve	+ ve	+ ve	+ ve	40
B. cereus	- ve	- ve	+ ve	+ ve	+ ve	40
E. coli	- ve	+ ve	+ ve	+ ve	+ ve	50
C. albicans	- ve	+ ve	+ ve	+ ve	+ ve	50
A.niger	- ve	- ve	+ ve	+ ve	+ ve	40
	Antimicrobial activity of isolated compound B					MIC
Microbes	50mg/ml	40mg/ml	30 mg/ml	20mg/ml	10mg/ml	
MRSA	- ve	- ve	- ve	- ve	+ ve	20
B. cereus	- ve	+ ve	+ ve	+ ve	+ ve	50
E. coli	- ve	- ve	- ve	- ve	+ ve	20
C. albicans	- ve	- ve	+ ve	+ ve	+ ve	40
A. niger	- ve	+ ve	+ ve	+ ve	+ ve	50

Key: + ve = microbial growth, - ve = absence of microbial growth

4.9. Interpretation of NMR Spectra for identification of Compounds A and B

^{13}C NMR and ^{1}H NMR for the two compounds were run in $CDCL_3$ (deuterated chloroform) at 400 MHz. Resulting ^{13}C NMR and ^{1}H NMR raw data was processed and used to generate COSY, HSQC and HMBC NMR. All the 1D and 2D NMR spectra have been appended in Appendix 1 and 2. Below is the interpretation of the spectra for compound A.

Table 4.12: Compound A: Interpretation of 1D NMR (^{13}C NMR, ^{1}H NMR) and 2D NMR (COSY, HSQC and HMBC) spectra

Carbon atom position	^{13}C NMR Chemical shifts[(δ)ppm] in ppm $CDCl_3$ at 400 MHz	^{1}H Proton NMR Chemical shifts [(δ)ppm] in $CDCl_3$ at 400 MHz	HMBC (^{13}C NMR-^{1}H NMR): ^{2}J and ^{3}J correlations.	C, H & O
		COSY(^{1}H NMR-^{1}H NMR)		
	HSQC (^{13}C NMR-^{1}H NMR) ^{1}J			
1	45.40	2.602 *d*, *J* = 16.8 Hz	C-2, C-3, C-5, C-10	C-H2
		4.077 *d*, *J* = 16.8 Hz	C-2, C-3, C-10	
2	197.32	*q*		C
3	134.07	*q*		C
4	143.95	*q*		C
5	150.84	*q*		C
6	120.87	6.610 *d*, *J* =7.2 Hz	C-8, C-10, C-18	C-H
7	130.98	7.360 *d*, *J* =7.2 Hz	C-5, C-9, C-14	C-H
8	125.32	*q*		C
9	119.52	*q*		C
10	30.41	*q*		C
11	146.15	*q*		C-OH
11-OH		7.525 *s*	C-9, C-11, C-12	
12	178.86	*q*		C
13	111.82	*q*		C
14	160.80	*q*		C-OH
14-OH		9.894s	C-8, C-13, C-14	
15	41.64	2.900, *d, J* =15.2 Hz	C-12, C-13, C-14, C-16	C-H2
		2.710, *dd* ,*J* = 15.2, 7.6 Hz	C-12, C-13,C-14, C-16	
16	79.39	3.660, *m*	C-13	C-H
17	17.70	1.190, 2.264, *d* = 6 Hz	C-15, C-16	C-H3
18	16.74	2.129, s	C-2, C-3, C-4	C-H3
19	11.97	1.946,s	C-3, C-4, C-5	C-H3
20	25.05	1.436, s	C-1, C-5, C-9, C-10	C-H3
21	56.12	3.393,s	C-16	-O-CH3

Key: *d* = doublet, *dd* = double doublet, s = singlet, *m* = multiplet, *J* = J coupling constant, q = quaternary carbon, ^{1}J = C & H atom single bond correlations, ^{2}J = C & H atom two bond correlations, ^{3}J = C & H atom three bond correlations

HSQC spectrum shows that carbon atom assigned position 1 (δ = 45.40 ppm) is a secondary aromatic carbon coupled via single bonds to two hydrogen atoms and the two hydrogen atoms on carbon 1 are also coupled across single bonds in the COSY spectrum (δ = 2.602, 4.077, *J* =16.8) (Appendix 1.3 and 1.4). Carbon 3, 4, 5, 8,9,11, 13, 14 are olefinic quaternary aromatic carbons, chemical shifts ranging from 110-170 ppm indicates carbon atoms in a benzene ring (Friebolin, 1993) and from the HSQC spectrum they are not attached to H atoms implying they are attached to other carbon atoms. C6 and C7 are olefinic tertiary aromatic carbons, from the HSQC spectrum both C6 and C7 are shown to be coupled with different H atoms across a single bond and the two H atoms in C6 and C7 have been shown to be coupled via a single bond in the COSY spectrum (δ = 6.10, 7.730, *J* =7.2) (Appendix 1.3 and 1.4). Although HSQC spectrum indicates that C11 and C14 are not coupled with H atoms via a single bonds, HMBC spectrum indicate that C11 and C14 are coupled with H atoms i.e. δ = 7.225 and 9.894 ppm respectively via a multiple bond and this can only imply C 11 and C 14 are attached to a hydroxyl (-OH) group (Appendix 1.5).

C15 is a secondary aliphatic carbon coupled via single bonds to two hydrogen atoms which are seen to be coupled across a single bond in the COSY spectrum (δ = 2.900, 2.700, *J* =15.2). Carbon 16 is an aliphatic carbon coupled with many H atoms (multiplet) and the nature of hydrogen splitting cannot be established because it is attached to a methoxy ($-OCH_3$) group. C17, 18, 19, 20 are aliphatic carbons whose chemical shifts are less that 20 ppm hence are attached to three H atoms forming a CH_3 groups; chemical shifts for primary carbon are less than 20 ppm (Friebolin, 1993). From the HSQC and HMBC spectrum, these carbon atoms have been shown to

be coupled with H atoms. HSQC shows C21 (δ = 56.12 ppm) is coupled to hydrogen atoms in a single split at 3.393 ppm which implies that this is a methoxy group. Methoxy groups are usually detected around 55-60 ppm (Friebolin, 1993). C2 (δ=197.32 ppm) can be ketone group (-CO) or an aldehyde group (COH) while C12 (δ=178.86 ppm) can either be a carboxylic group (-COOH), -CO or –COH group according to 13 C NMR chemical shifts standard reference table (Friebolin, 1993). If C2 and C12 were coupled with H atoms through O, HMBC will be able to show that coupling but it does not indicate any coupling between H atoms attached to O on C12 and the adjacent carbon atoms. This implies C2 and C12 are attached to oxygen only forming ketone groups. From the HMBC spectrum H atoms on carbon 1, 6, 7 11, 14, 15, 16, 17, 18, 19, 20, 21 have been shown to be coupled to other carbons across two (^{2}J) and three bonds (^{3}J) as indicated in Table 4.12. High resolution mass spectrum for compound A (Appendix 1.6) shows that the molecular ion (M+) was detected at 357.1700 m/z and fits in the structure and molecular formula of the compound below.

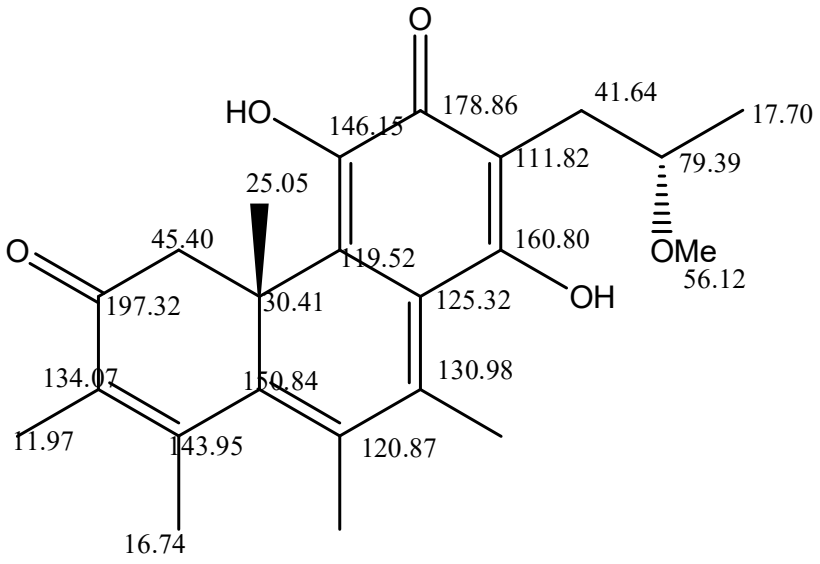


Figure 4.33: C13 NMR Chemical shifts for Compound A

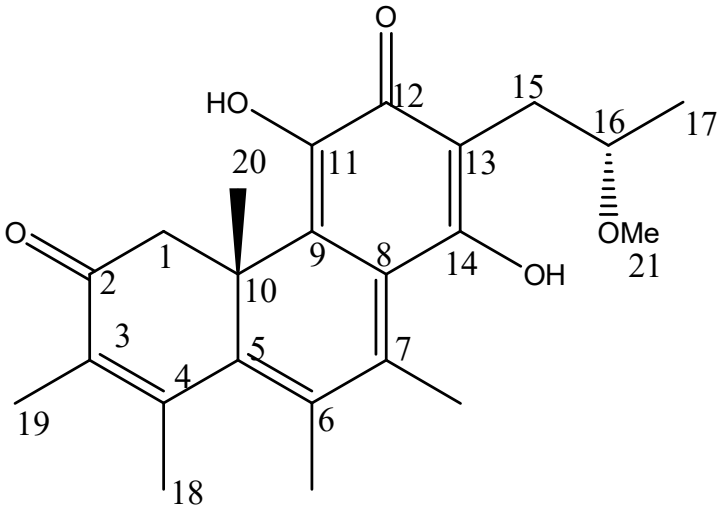
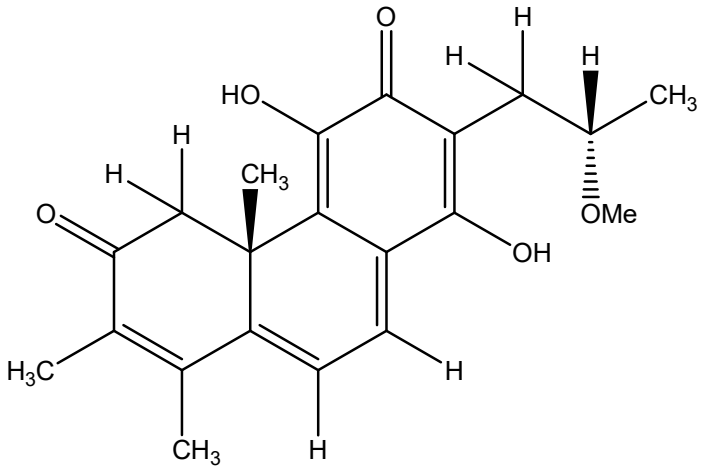


Figure 4.34: Carbon atom positions on compound A

Compound A was identified as (S) - 4, 4 a – dihydro - 5, 8 – dihydroxy – 7 - ((S) – 2 - methoxypropyl) - 1, 2, 4 a –trimethylphenanthrene -3, 6-dione.



$C_{21}H_{24}O_5$
Exact Mass: 356.1624
Mol. Wt.: 356.4123
m/e: 356.1624 (100.0%), 357.1657 (22.7%), 358.1691 (2.5%), 358.1666 (1.0%)

Figure 4.35: Compound A: (S) - 4, 4 a – dihydro - 5, 8 – dihydroxy – 7 - ((S) – 2 - methoxypropyl) - 1, 2, 4 a –trimethylphenanthrene -3, 6-dione

Table 4.13: Compound B: Interpretation of 1D NMR (13 CNMR, 1H NMR) and 2D NMR (COSY, HSQC and HMBC) spectra.

Carbon atom position	^{13}C NMR Chemical shifts [(δ)ppm] in ppm $CDCl_3$ at 400 MHz	^{1}H Proton NMR Chemical shifts [(δ)ppm] in $CDCl_3$ at 400 MHz	HMBC (^{13}C NMR-^{1}H NMR): ^{2}J and ^{3}J correlations.	C, H & O
		COSY(^{1}H NMR-^{1}H NMR)		
	HSQC (^{13}C NMR-^{1}H NMR) ^{1}J			
1	45.41	2.613 *d*, *J* = 16.8 Hz	C-2, C-3, C-5, C-10	C-H2
		4.089 *d*, *J* = 16.8 Hz	C-2, C-3, C-10	
2	197.43	*q*		C
3	134.15	*q*		C
4	143.95	*q*		C
5	151.09	*q*		C
6	120.88	6.617 *d*, *J*=6.8 Hz	C-8, C-10, C-18	C-H
7	130.99	7.373 *d*, *J*=6.8 Hz	C-5, C-9, C-14	C-H
8	125.19	*q*		C
9	119.55	*q*		C
10	30.43	*q*		C
11	146.15	*q*		C-OH
11-OH		7.515 *s*	C-9, C-11, C-12	
12	179.12	*q*		C
13	111.84	*q*		C
14	160.81	*q*		C-OH
14-OH		9.910s	C-8, C-13, C-14	
15	41.66	2.918, *d, J*=14.8 Hz	C-12, C-13, C-14, C-16	C-H2
		2.699, *dd ,J* = 14.8, 7.6 Hz	C-12, C-13,C-14, C-16	
16	79.43	3.664, *m*	C-13	C-H
17	17.73	1.185, *d*, *J*=6	C-15, C-16	C-H3
18	16.78	2.138 s	C-2, C-3, C-4	C-H3
19	12.01	1.957 s	C-3, C-4, C-5	C-H3
20	25.05	1.446 s	C-1, C-5, C-9, C-10	C-H3
21	56.15	3.402 s	C-16	-O-CH3

Key: *d* = doublet, *dd* = double doublet, s = singlet, *m* = multiplet, *J* = J coupling constant, q = quaternary carbon, ^{1}J = C & H atom single bond correlations, ^{2}J = C & H atom two bond correlations, ^{3}J = C & H atom three bond correlations

C1 (δ = 45.41ppm) is a secondary aromatic carbon coupled via single bonds to two hydrogen atoms as indicated by the HSQC spectrum and the two hydrogen atoms on C1 are also coupled across single bonds in the COSY spectrum (δ = 2.613, 4.089, *J* =16.8) (Appendix 2.3 and 2.4). Carbon 3, 4, 5, 8, 9, 11, 13, 14 are olefinic quaternary aromatic carbons, chemical shifts ranging from 110-170 ppm and indicate carbon atoms in a benzene ring (Friebolin, 1993). C6 and C7 are olefinic tertiary aromatic carbons, from the HSQC spectrum both C6 and C7 are shown to be coupled with a different H atom across single bonds and the two H atoms in C6 and C7 are coupled via a single bond in the COSY spectrum (δ = 6.617, 7.373, *J* =6.8) (Appendix 2.3 and 2.4). HMBC spectrum indicate that C11 and C14 are coupled with H atoms i.e. δ = 7.515 and 9.910 ppm respectively via a multiple bond and this can only imply C 11 and C 14 are attached to a hydroxyl (-OH) group because HSQC does not show single bond correlation between these carbon atoms and H atoms (Appendix 2.5).

C15 is a secondary aliphatic carbon coupled via single bonds to two hydrogen atoms which are seen to be coupled across a single bond in the COSY spectrum (δ = 2.918, 2.699, *J* =14.8). Carbon 16 is an aliphatic carbon coupled with many H atoms (multiplet). C17, 18, 19, 20 are aliphatic carbons whose chemical shifts are less that 20 ppm hence are CH_3 groups (Friebolin, 1993). HSQC shows C21 (δ = 56.15 ppm) is coupled to hydrogen atoms indicated by a single split at 3.402 ppm which implies that this is a methoxy group (Appendix 2.3 and 2.4). Both C2 (δ=197.43 ppm) and C12 (δ=179.12ppm) are ketone groups because HMBC does not indicate any coupling between H atoms attached to O on C12 and the adjacent carbon atoms implying C2 and C12 are attached to oxygen only forming ketone groups (Appendix 2.5). From the HMBC spectrum H atoms on carbon 1, 6, 7 11, 14, 15, 16, 17, 18, 19, 20, 21 have been shown to be coupled to other carbons across two (^2J) and three bonds (^3J) as indicated in Table 4.13. High

resolution mass spectrum for compound B (Appendix 2.6) shows that the molecular ion (M+) was detected at 357.1691 m/z and fits in the structure and molecular formula of the compound B below.

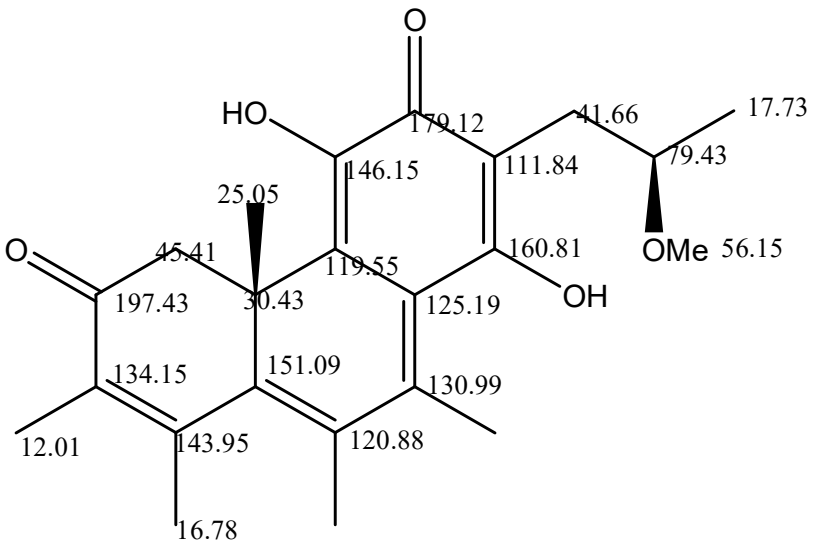


Figure 4.36: C13 NMR Chemical shifts for compound B

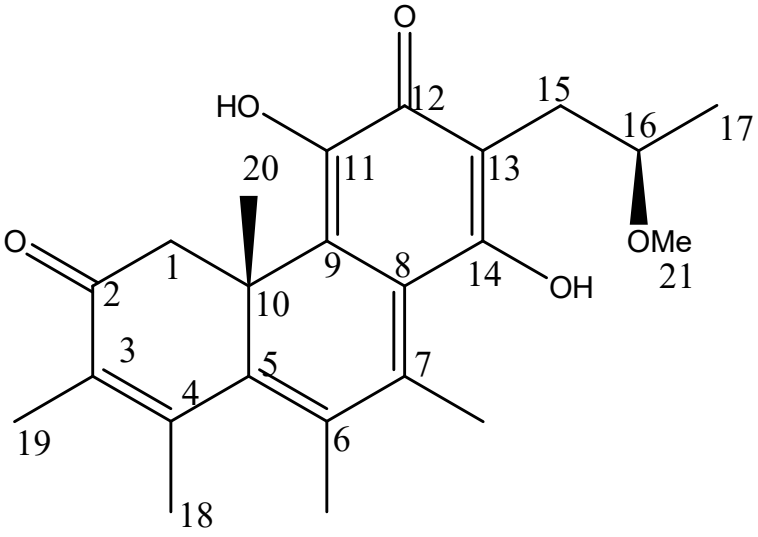
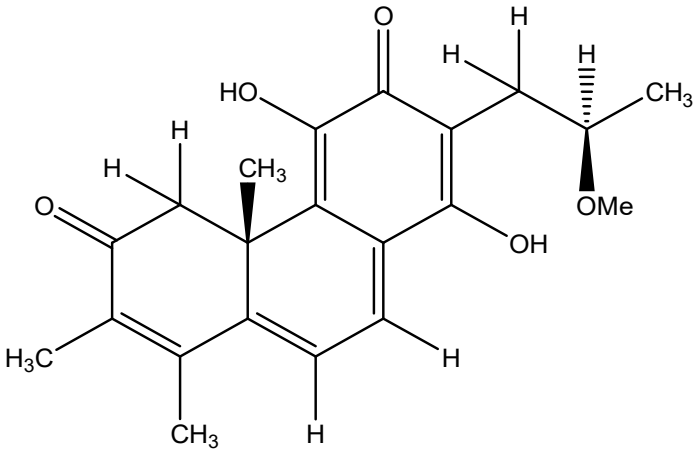


Figure 4.37: Carbon atom positions on compound B

Compound B was identified as (S)-4, 4 a – dihydro - 5, 8-dihydroxy - 7 - ((R) – 2 - methoxypropyl) -1, 2, 4a - trimethylphenanthrene -3, 6-dione.



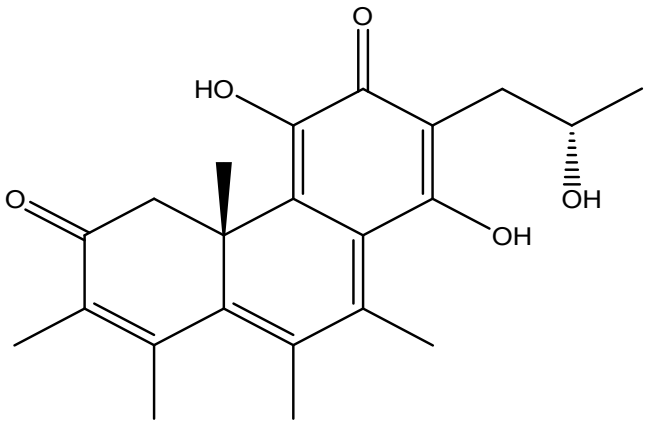
$C_{21}H_{24}O_5$
Exact Mass: 356.1624
Mol. Wt.: 356.4123
m/e: 356.1624 (100.0%), 357.1657 (22.7%), 358.1691 (2.5%), 358.1666 (1.0%)

Figure 4.38: Compound B: (S)-4, 4 a – dihydro - 5, 8-dihydroxy - 7 - ((R) – 2 - methoxypropyl) - 1, 2, 4a - trimethylphenanthrene -3, 6-dione.

4.10. Comparison of Compound A and B

Both compounds have been shown to have similar structure and have the same molecular formula as it has been established from ^{13}C NMR , ^{1}H NMR chemical shifts (δs) and MS mass to charge ratios (m/z) which are also similar. The respective NMR chemical shifts are varying by decimals in the two compounds. However, TLC profiles indicated that compound A and B had different Rf values i.e 0.500 and 0.375 respectively. The only difference between the two compounds is the stereochemistry on the aliphatic side chain where there is a methoxy group attached, the three benzene rings form the basic biogenetic precursor of the class which the compounds belong to. Hence A and B are R and S forms of the same compound. Both stereoisomers (R and S forms of the compound) can be simply referred using one name as 4, 4a-

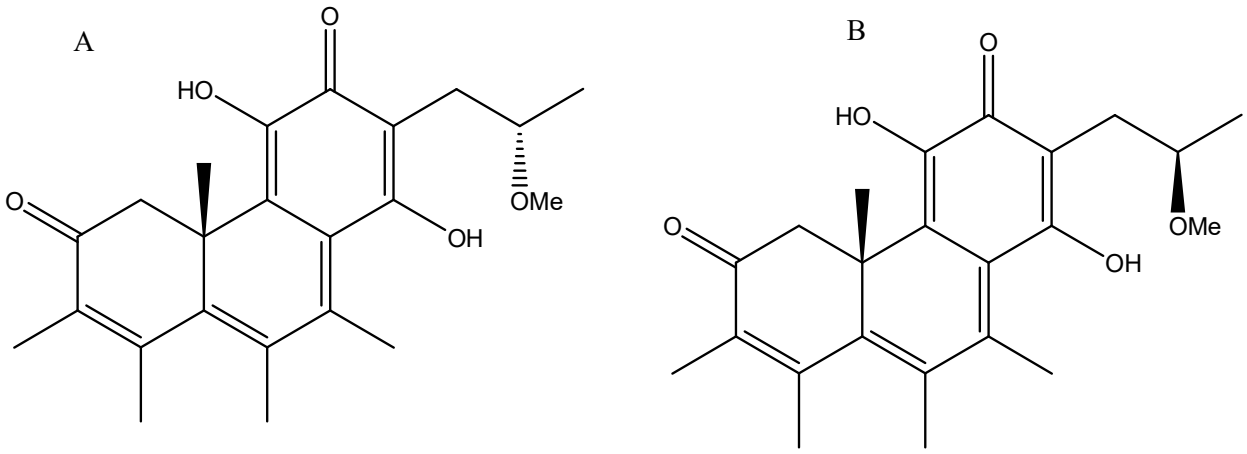
dihydro-5, 8-dihydroxy-7-(2-methoxypropyl)-1, 2, 4a-trimethylphenanthrene-3, 6-dione. Through literature and compound identity search in SciFinder® and ChemFinder database, the compound is a new compound and is new abietane diterpene which is chemically similar to Coleon E (a diterpene which has been isolated from *Plectranthus barbatus*). 4, 4a-dihydro-5, 8-dihydroxy-7-(2-methoxypropyl)-1, 2, 4a-trimethylphenanthrene-3, 6-dione differs from Coleon E in the aliphatic side chain where there is a hydroxyl group attached on Carbon 16 (forming a hydroxypropyl side chain) rather than a methoxy group (forming a methoxyprophyl side chain in the compounds identified). Below is the structure of Coleon E (Figure 4.39).



Coleon E (Yulianto, *et al*., 2016, Porfirio *et al*., 2010, Fale *et al*, 2009)

Figure 4.39: Structure of Coleon E

Because of the close relationship of Compound A and B to Coleon E. Compound A can be simply referred to as (16S)-MethoxyColeon E while Compound B can likewise be referred to as (16R)-MethoxyColeon E.



(16S)-MethoxyColeon E

(16R)-MethoxyColeon E

4,4a-dihydro-5,8-dihydroxy-7-(2-methoxypropyl)-1,2,4a-trimethylphenanthrene-3,6-dione

Figure 4.40: (16S) -MethoxyColeon E and (16R) MethoxyColeon E

CHAPTER FIVE: DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1. Discussion

Based on the current study, *MatK* and *Rbcl* genes from ten *Plectranthus* species have been sequenced and phylogenetic trees have been reconstructed from the sequences. Based on *MatK* gene sequences, *P.caninus, P.otostegioides, P. barbatus* and *P. lanuginosus* have been grouped together (Cluster A) while *P.ornatus*, *P. pseudomarrubioides* and *P.eagyptiacus* have been grouped together in another group; Cluster B (Figure 4.5). Species grouped together imply that they share a close common ancestor. The phylogenetic tree also shows that *P.montanous* and *P.amboinicus* form another group (Cluster C) and all the species share a distance common ancestor with *P.edulis.* On the other hand, a phylogenetic tree reconstructed based on the *Rbcl* gene sequences grouped together *P. pseudomarrubioides, P.montanous, P.ornatus, P.amboinicus* and *P. edulis* (Cluster D) indicating that these four species are closely related (Figure 4.6). The *Rbcl* phylogenetic tree also grouped together *P. caninus, P.lanuginosus, P.barbatus* and *P. otostegioides* forming another group (Cluster E) and also indicates that both groups are related to *P.aegyptiacus* which is grouped alone. From the results, it may be argued that *MatK* gene is better in delimiting *P.aegyptiacus* and likewise *Rbcl* gene is better in delimiting *P.edulis* among species within the *Coleus* clade.

Comparison of the two trees based on *MatK* and *Rbcl* analyses show that they are very similar. For example based on the *MatK* genes; *P. caninus*, *P. otostegioides, P. barbatus* and *P. lanuginosus* have been grouped together. The same pattern can be observed in the phylogenetic tree created using the *Rbcl* genes implying Cluster A based on *MatK* genes is similar to Cluster E based on *Rbcl* genes. Again based on the *MatK* genes; *P. pseudomarrubioides*, *P. ornatus*, *P. montanous* and *P. amboinicus* have been shown to be closely related. This close relationship of

these species can also be observed in phylogenetic tree produced using the *Rbcl* genes which likewise implies Cluster B based on *MatK* genes is similar to Cluster D based on *Rbcl* genes (Figure 4.7). Based on *MatK* genes, *P.aegyptiacus* was shown to be closely related to *P. pseudomarrubioides* and *P.ornatus* while based on *Rbcl* genes, *P.edulis* is closely related to *P.amboinicus, P. ornatus, P. pseudomarrubioides, P. amboinicus* and *P. montanous.* Generally species grouped together in phylogenetic trees imply that they share a more recent ancestor.

Plectranthus species have been classified based on their ethnobotanical uses by Lukhoba *et al*. (2006). In that study, the authors were able to assign all the *Plectranthus* species in this study to the *Coleus* clade. Further, *P.montanous* grouped together with *P. pseudomarrubioides*, *P.amboinicus* was grouped together with *P.aegyptiacus* while *P.barbatus, P.caninus* and *P. lanuginosus* were grouped together. Their findings are similar to the observation in this study based on *MatK* and *Rbcl* gene sequences. *P.montanous* and *P.pseudomarrubioides* have been shown to be closely related and *P.barbatus*, *P.caninus* and *P. lanuginosus* have been shown to be closely related as well and hence have been grouped together.

P. amboinicus grouped together with *P. montanous* was shown to be distantly related to *P. barbatus* based on phylogenetic trees reconstructed from the following three plastid DNA gene sequences: *trnL* intron, *trnL-trnF* intergene spacer and *rps16* gene (Paton *et al*., 2004). In addition, Al-Qurainy *et al*. (2014) likewise observed that *P. amboinicus* was closely related to *P. montanous*. Such findings are supported by the results in the current study where by *MatK* and *Rbcl* phylogenetic trees showed that *P. amboinicus* was closely related to *P. montanous* but distantly related to *P. barbatus.* A phylogenetic tree reconstructed by Al-Qurainy *et al*. (2014) based on *nrITS* gene also indicated that *P.barbatus* was closely related to *P. caninus*. The findings by Paton *et al*. (2004) and Al-Qurainy *et al*. (2014) are in line with data in the current study based on the

MatK and *Rbcl* which have grouped together *P.amboinicus* and *P.montanous* while also *P.barbatus* to *P.caninus* implying that these pairs of species are closely related.

A dendrogram produced from cluster analysis of morphological characters described by Paton *et al.* (2009) in the Flora of Tropical East Africa (Figure 4.18) showed two main groups; Cluster H and Cluster I. Cluster H contained the species *P. lanuginosus, P. edulis, P. barbatus, P. caninus*, *P. otostegioides* and *P. ornatus* while Cluster I contained *P. montanous, P. amboinicus, P. pseudomarrubioides* and *P. aegyptiacus*. Inner branches of Cluster H further showed *P. lanuginosus, P. edulis, P. barbatus* were morphologically similar and *P. caninus*, *P. otostegioides* and *P. ornatus* are morphologically similar as well hence have been grouped together. Morphological similarity in most cases is correlated to genetic similarity (Gottlieb, 1981). Hence, these close relations based on morphology within each group implied that members within each group were closely related. Such relationships of *Plectranthus* species described in the FTEA were very similar to what have has been observed from the phylogenetic trees created from *MatK* and *Rbcl* gene sequences in the current study.

Grayer *et al*. (2010) investigated the distribution of exudate flavonoids in *Plectranthus* species and consequently constructed a phylogenetic tree based on the distribution. Of particular interest to the current study is *P. aegyptiacus, P. pseudomarrubioides* and *P. montanous* which are among the *Plectranthus* species investigated by Grayer *et al*. (2010). The study reported that both *P. pseudomarrubioides* and *P. montanous* produce Quercetin 3-methyl ether and Quercetin 3, 7-dimethyl ether. Results from Grayer *et al*. (2010) also reported that both *P. pseudomarrubioides* and *P. aegyptiacus* produced Cirsimaritin. This implies that *P. pseudomarrubioides* is genetically similar to both *P. aegyptiacus* and *P.montanous* and this supports the findings of the current study.

As at 2016 (NCBI Nucleotide Database, 2016), 18 *Rbcl* gene sequences and 26 *MatK* gene sequences of different *Plectranthus* species had been deposited in the genebank at NCBI by various authors as at 2016 (NCBI Nucleotide Database, 2016). By comparing the *Plectranthus MatK* and *Rbcl* gene sequences of the studied *Plectranthus* species with the already deposited NCBI Gene Bank *MatK* and *Rbcl* gene sequences of other *Plectranthus* species, it was found that *P. amboinicus* was related to *P. fruticosus, P. zeylanicus* and *P. prostratus* while *P. montanous* was related to *P. mollis* and *P. malabricus* based on *MatK* gene sequences. In addition, based on the *Rbcl* sequences, *P. ornatus* was related to *P. parviflorus* and *P. amicorum* , *P. edulis, P. montanous* and *P. pseudomarrubioides* were related to *P. prostratus* while *P. aegyptiacus* and *Plectranthus hadiensis var. tomentosus* (Benth. ex Mey.) Codd were likewise related to each other (Figure 4.8 and 4.9).

One advantage of comparing the obtained *MatK* and *Rbcl* gene sequences with the results from the Genbank, was the possibility of ascertaining the identity of the ten species under study so long as their *MatK* and *Rbcl* gene sequences had already been deposited in the gene bank. For example the *MatK* and *Rbcl* gene sequences for *P.amboinicus, P. barbatus* and *P. caninus* had already been deposited in the genbank by previous researchers. It was therefore possible to perform a sequence similarity search in the Genbank using *MatK* an *Rbcl* gene sequences from the ten species as querry sequences which gave a sequency similarity match of >99% for the sequences already available in the Genbank. Such a search will also give the name of the species which was given when the sequence was being deposited in the gene database by the author. Hence species such as *P.barbatus, P.caninus* and *P.ambonicus* in addition from being identified by a plant taxonomist, they were also identified through a sequence similary search using Basic Local Allignment Search (BLAST) tool in the gene bank. A phylogenetic tree containing two sequences

of the same gene of a particular species will show the sequences/branches terminating at near or the same point if the sequences were near or 100% similar (Figure 4.8 and 4.9). Sequence similarity search using BLAST may help to corroborate data from morphology and other sources and may also aid in certifying identity of medicnal plant material.

Leaf micromorphology in the present study involved investigation of the internal leaf anatomy and stomatal distribution of the ten *Plectranthus* species investigated. Such a study focused on the arrangement of the leaf mesophylls, type of epidermal cells, trichomes, stomata density, stomata type as well as stomata occurrence on both the abaxial and adaxial surfaces of the leaves. Data obtained from the study was used to generate a dendrogram for better understanding of the taxonomic relationships between the ten species. Leaf anatomical characters which contributed to most of the differences in the ten *Plectranthus* investigated included: epidermal cell type polygonal or sinuous, trichome type which ranged from being long stalked, short stalked, glandular, non glandular to capitate, nature of the leaf mesophyll which was either dorsivental or homogeneous and nature of the palisade layer (Figure 4.12 to Figure 4.16). From the leaf micromorphology dendrogram obtained after clustering the anatomical characters (Figure 4.7), the ten *Plectranthus* species were grouped into two main groups; *P.caninus, P.ornatus, Potostegioides, P.montanous* and *P. pseudomarrubioides* were grouped together in Cluster F while *P. aegyptiacus, P.amboinicus, P.edulis, P.barbatus* and *P.lanuginosus* were placed in Cluster G. The inner branches of the Cluster F show that *P. caninus* is similar to *P.ornatus.* In addition by looking at Cluster F, *P.otostegioides*, *P.montanous* and *P.pseudomarrubioides* were found to be morphologically similar. *P.aegyptiacus* was also placed close to *P.ambonicus* and lastly *P.edulis, P. barbatus* and *P. lanuginosus* were grouped together as well as seen in Cluster G.

Epidermal cell type, type of stomata, type of trichome and nature of the mesophyll have been used before by Duarte and Lopes (2007) to differentiate *P.barbatus* and *P. neochilus* from other South American Labiatae species referred by the same common name. *P.barbatus* was reported to possess anomocytic stomata, dorsiventral mesophyll, capitate glandular trichomes and polygonal epidermal cells (Duarte and Lopes, 2007). Such findings on leaf anatomical characters of *P.barbatus* have been supported by the results of the current study. Four types of glandular trichomes of taxonomic significance on the abaxial surface of leaves have been reported in various developmental stages of *P.ornatus*. These include long stalked capitate trichomes, short stalked capiate trichome, digitiform and conoidal trichomes (Ascensão *et al*., 1999) and this supports the current study whereby *P.ornatus* was found to posses long-stalked glandular trichomes.

Khalik (2016) reported that *P.montanous* and *P.pseudomarrubioides* had short stalked peltate trichomes and this likewise corrobotates the current leaf micromorphology study whereby the two species have been reported to have short stalked trichomes. A study by Ascensao *et al*. (1998) revealed that *P.madagascariensis* has both capitate glandular trichomes responsible for the production of various kinds of essential oils. *P. amboinicus* was reported to have rectangular to polygonal leaf epidermal cells with more grandular than non glandular trichomes on both the adaxial and abaxial surface (Kaliappan and Viswanathan, 2008). In addition, the leaf mesophyll of *P.amboinicus* was also described to be homogeneous consisting of undifferentiated compact squarish cells (Kaliappan and Viswanathan, 2008). These results of leaf micromorphology of *P.amboinicus* were in agreement with the results obtained in the current study whereby *P. amboinicus* was reported to have homogeneous mesophyll and short-stalked trichomes. Generally, isobilateral, centric or dorsiventral leaf mesophyll have been reported in species within the Labiatae family (Fritsch *et al*., 1950).

Organisms in a dendrogram grouped together imply that they are more similar morphologically. In most cases similarity based on morphology is related to genetical similarity because traits are determined by genes and a similar character in different plants strongly suggests that there is a shared gene by those species possessing the morphological character. Hence it can be assumed that, the more close the species are grouped may suggest the more recently they diverged from a common ancestor. Morphological characters are oftenly affected by environmental factors and morphological variation seen in species can either be environmentally induced or genetically based (Klingenberg, 2010). The same species growing in different ecological zones can display varied morphological characters. For example, by referring to the leaf micromorphology dendrogram, Cluster F shows that *P. otostegioides*, *P. montanous* and *P. pseudomarrubioides* have been grouped together and *P. edulis, P. barbatus* and *P. lanuginosus* have been grouped together as well in Cluster G. Such a grouping based on character similarly can be attributed to environmental influence on the species growing in their habitats. *P. otostegioides*, *P. montanous* and *P. pseudomarrubioides* were collected in dry regions of Kenya i.e Olorgesailie, Makueni and Naivasha respectively while *P.edulis, P.barbatus* and *P.lanuginosus* were collected from wet regions in Kenya i.e Trans Nzoia, Nairobi and Nairobi respectively. This may strongly suggest that environment might have influenced their stomata density and other leaf micromorphology characters making the species to possess near similar characters hence after cluster analysis they were grouped together.

As mentioned before, a dendrogram produced from cluster analysis of gross morphological characters described by Paton *et al.* (2009) in the Flora of Tropical East Africa (Figure 4.18) groups the ten *Plectranthus* species into two main groups; Cluster H and Cluster I. By comparing the gross morphological dendrogram (Cluster H and I) with the leaf micromorphology dendrogram (Cluster

F and G), some similarities between the two can be deduced. For instance, *P. lanuginosus, P. edulis, P. barbatus* have been grouped together by the two dendrograms (Cluster G and H). Similary, *P.montanous* and *P.pseudomarrubioides* have been grouped together in the two dendrograms (Cluster F and I).

Comparison of the leaf micromorphology dendrogram and the *MatK* and *Rbcl* phylogenetic trees has been done on Figure 4.19. One major similarity between the leaf micromorphology dendrogram and the molecular phylogenetic trees include placement of *P.barbatus* and *P. lanuginosus* in the same group. Other species which have been placed in the same group by both the obtained leaf micromorphology dendrogram and the molecular phylogenetic trees are *P. pseudomarrubioides* and *P. montanous* and *P.ornatus*. This clearly shows that the obtained leaf micomorphology data is suppoted by molecular data from *MatK* and *Rbcl* sequences. This implies that *P. barbatus* and *P. lanuginosus* are closely related and the same conclusion of close relatedness can be made for *P. pseudomarrubioides*, *P. montanous* and *P. ornatus.*

Findings on the leaf micromorphology study are supported by studies conducted by Lukhoba *et al*. (2006) whereby *Plectranthus* species were classified based on their ethnobotanical uses. From the study, the authors were able to group most of the species belonging to *Coleus* clade together. *P. montanous* was placed in the same group with *P. pseudomarrubioides*; *P. amboinicus* was placed together with *P. aegyptiacus* while *P.barbatus, P.caninus* and *P. lanuginosus* were grouped together as well. A study carried out by Khalik (2016) using ultrastructure characters of pollen grains and trichomes to classify Saudi Arbaian *Plectranthus* species showed that *P. montanous* and *P. pseudomarrubioides* were closely related to each other and this is in agreement with the current study whereby the two species have been grouped together.

Morphological characters have been employed widely in the identification and classification of plants. In closely related species, these morphological characters may differ by a small margin and it may not be easy to identify and classify closely related species. Studies on leaf micromorphology are important sources of taxonomic characters for classification of plants which are difficult to delimit. Some of these characters are of diagnostic value and have been used successfully in infrageneric and interspecific classification in plants. Most morphological characters overlap at species level and at the lower taxonomic ranks especially in genus and sub-genus levels making it hard to classify species based on the general morphological characters. As a result diagnostic morphological characters are vital when it comes to morphology based phylogenetic studies. Micromorphological characters are examples of such diagnostic characters which have proved vital in the delimitation of species below the genus level (Salmaki *et al*., 2009; Xiang *et al*., 2010).

Based on the current leaf micromorphology study epidermal cell type, type and nature of trichomes, mesophyll type and palisade layer have proved to be vital diagnostic characters for differentiating the ten species investigated while characters such as the presence or absence of a succulency, type of stomata, number of stomata and occurrence of stomata have been shown to be less important in differentiating the ten species. Dendrogram produced based on the leaf micromorphological characters classifies the ten *Plectranthus* species in a pattern which has been supported from previous research. Hence the current study has proved that studies of leaf micromorphology can aid in determination of diagnostic morphological characters which can aid in plant delimitation and phylogenetic studies.

With regard to molecular phylogeny of the ten *Plectranthus* species in the *Coleus* clade, phylogenetic trees reconstructed based on *MatK* and *Rbcl* gene sequences showed phylogenetic

relationships among the ten species and proved that the ten species are actually related. Furthermore, the results obtained were in congruent with results obtained by previous resarchers on phylogeny of the *Plectranthus* genus as far as the ten species are concerned. The study proved that molecular characters are indeed valuable in phylogenetic studies especially in the lower taxonomic ranks whereby morphological characters may not delimit species successfully.

Majority of *Plectranthus* species have been used in ethnomedicine for the management of various disease conditions. Bioassay studies have identified several members from the genus with promising bioactivities while phytochemical analysis have led to the isolation of important secondary metabolites from *Plectranthus* species (Waldia *et al*., 2011). In the current study, crude organic extracts from all the ten species were tested against three bacteria (MRSA, *B.cereus, E. coli*) and two fungi (*C.albicans* and *A.niger*).). Leaf crude organic DCM: MeOH (1:1) from *P. barbatus*, *P. lanuginosus* and *P. ornatus* were found to possess higher antibacterial activity compared to the other species against the microbes investigated. *P.barbatus* had the highest activity with respect to the other species investigated and was subsequently subjected to phytochemical analysis which led to isolation of two pure compounds with antimicrobial activity.

In the present study, all the crude organic extracts from the ten *Plectranthus* species were found to have varying antibacterial activity against MRSA with *P.lanuginosus* and *P.barbatus* leaf crude extracts exhibited a higher inhibition on the growth of MRSA compared to the other *Plectranthus* species. MIC values of *P.barbatus* and *P.lanuginosus* leaf crude extracts against MRSA were 25mg/ml and 40 mg/ml respectively. When the crude organic extracts were tested against *B. cereus*, highest growth inhibition was observed in *P. otostegioides* (MIC value = 100mg/ml), *P.lanuginosus* (MIC value = 40 mg/ml) and *P.barbatus* (MIC value = 25 mg/ml), while only crude extracts of *P.barbatus* (MIC value = 100 mg/ml) and *P.edulis* (MIC value = 100

mg/ml) inhibited the multiplication of *E.coli*. The other species did not inhibit the growth of *E.coli.* On the other hand, when the crude organic extracts were subjected to *C. albicans* to assess their antifungal activity, *P.otostegioides, P.ornatus, P.aegyptiacus, P.barbatus* and *P.amboinicus* were found to inhibit the growth of *C.albicans,* highest growth inhibition of *C.albicans* being observed under *P. ornatus* (MIC value = 50 mg/ml) and *P. barbatus* (MIC value = 50 mg/ml). Lastly, growth inhibition of *A.niger* was observed only in the crude organic extracts of *P. lanuginosus* (MIC value = 50 mg/ml) and *P.barbatus* (MIC value = 100 mg/ml)

Multiple comparisons were carried out using Dunnett T test whereby various concentrations of the DCM: MeOH leaf crude extracts of the *Plectranthus* species were compared with the positive controls. Comparison of the growth inhibitions of 200 mg/ml of both *P. barbatus* and *P.lanuginosus* leaf crude extracts against MRSA with growth inhibitions of 50 mg/ml of Amoxicillin against MRSA gave a significance level of 0.451 and 0.439 respectively (Table 4.6). Hence, it was concluded that growth inhibition of MRSA due to 200 mg/ml of both *P. barbatus* and *P. lanuginosus* leaf crude extracts was not significantly different from the growth inhibition of MRSA due to Amoxicillin at significance level (p) of ≤ 0.05. With respect to antifungal activity, various concentrations of the DCM: MeOH leaf crude extracts of *Plectranthus* species were likewise compared with Ketoconazole. Comparison of growth inhibition of 200 mg/ml of both leaf crude extracts of *P.ornatus* and *P. barbatus* against *C.albicans* with growth inhibition of 40 mg/ml of Ketoconazole against *C.albicans* gave a significance level of 0.291 and 1.00 respectively (Table 4.6). This implied that *C.albicans* growth inhibition by 200 mg/ml of *P. ornatus* and *P.barbatus* leaf crude extracts was not significantly different from *C.albicans* growth inhibition by 40 mg/ml of Ketoconazole at $p \leq 0.05$.

From the preliminary screening of DCM: MeOH leaf crude extracts from the ten *Plectranthus* species for antimicrobial activity through disc diffusion, *P. barbatus* displayed the highest growth inhibition zones against the test microbes compared to the other species under study. Upon determination of MICs of the DCM: MeOH crude extracts from the ten *Plectranthus* species using broth dilution, it was also observed that *P.barbatus* displayed the lowest MIC values against the microbes. MIC values of the leaf crude extracts of *P. barbatus* against MRSA, *B.cereus, E.coli, E.albicans* and *A.niger* were as follows: 25 mg/ml, 40mg/ml, 100 mg/ml, 50 mg/ml and 100 mg/ml respectively (Table 4.7). Hence, it was concluded that *P. barbatus* was better in inhibiting the growth of the microbes under study compared to the other nine species. Consequently, it was subjected to further antimicrobial testing whereby leaf crude extracts of *P.barbatus* were fractionated using five solvents of increasing polarity namely: petroleum ether, n-hexane, DCM, chloroform and ethyl acetate. These fractionated crude extracts were tested against each of the five microbes using disc diffusion and broth dilution techniques.

Antimicrobial activity of *P.barbatus* fractions indicated that, DCM (MIC value = 40mg/ml) and hexane (MIC value = 150mg/ml) leaf crude extracts of *P.barbatus* were found to inhibit the growth of MRSA while petroleum ether, chloroform and ethyl acetate crude extracts had no activity against MRSA. Secondly, petroleum ether (MIC value = 75mg/ml), DCM (MIC value = 150 mg/ml), chloroform (MIC value = 75 mg/ml) and EtoAC (MIC value = 60 mg/ml) leaf crude extracts of *P.barbatus* and inhibited the multiplication of *B.cereus*. Likewise, when all the crude extracts of *P.barbatus* were tested against *E.coli*, they all inhibited the growth of *E.coli* with DCM (MIC value = 25 mg/ml) and Chloroform (MIC value = 25 mg/ml) crude extracts displaying the highest growth inhibition. On antifungal activity, *C.albicans* growth was likewise inhibited by all *P.barbatus* leaf crude extracts with DCM (MIC value = 25 mg/ml) and chloroform

(MIC value = 25 mg/ml) extracts inhibiting the growth most. Lastly, only two leaf crude extracts of *P.barbatus* inhibited the growth of *A.niger* which were the Hexane (MIC value = 150 mg/ml) and DCM (MIC value = 75 mg/ml) crude extracts. In general, DCM fraction of *P. barbatus* displayed the lowest MIC values was assumed to be better in inhibiting the multiplication of the test microbes compared to the other nine *Plectranthus* species.

From the multiple comparisons of growth inhibitions of various concentrations of leaf crude partitions of *P.barbatus* with that of the respective positive control using Dunnet T test, it was observed that the comparison of antifungal activity/inhibition zone of 200 mg/ml of the DCM crude extract against *C.albicans* with Ketoconzole activity gave a significance level of 0.109 (Table 4.9). This implied that the 200 mg/ml of the DCM *P.barbatus* leaf crude extract had anti-candida activity which was not significantly different from that of Ketoconazole at $p \leq 0.05$. However all the other crude extracts of *P.barbatus* had antimicrobial activities against the microbes tested only that the activities were low and significantly different from the positive control activities at $p \leq 0.05$ (Table 4.9). Bioactive monoterpenes, sesquiterpenes, diterpenes and phenolic compounds have been reported in *Pletranthus* genus (Gaspar-Marques *et al*., 2004). Hence, it can be concluded that at the tested concentrations, the observed antimicrobial activity of crude extracts of the study *Pletranthus* species and partitions of *P.barbatus* could be due to non-specific interactions amongst the aforementioned biomolecules.

Various previous studies have reported the presence of antimicrobial activity in *Plectranthus* species. For example, crude ethanol extracts of *P. barbatus* have been shown to possess antimicrobial activity against *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus pneumonia, E. coli* (Veríssimo *et al*., 2014). Aqueous methanol and hexane extracts of *P. barbatus* have likewise been reported to have antimicrobial activity against *S. aureus* (Matu and van Stedan,

2003). Hence these previous studies by Veríssimo *et al*., (2014) and Matu and van Stedan (2003) support the findings of the current study on *P. barbatus* which has been shown to inhibit the growth of *S. aureus*. Elsewhere methanolic extracts from the roots of *P. barbatus* were also reported to possess strong anti-candida activity (Runyoro *et al*, 2006) which is also in line with the findings of the current study. Aqueous extracts of *P. barbatus* have also been reported to have antibacterial activity against *Streptococcus sobrinus* and *Streptococcus mutans* (Figueiredo *et al*., 2010) while methanolic extracts of *P.barbatus* and *P. amboinicus* have been reported to have antifungal activity against *Candida krusei* (Tempone *et al*., 2008). Other findings corroborating the current study findings on *P. barbatus* fractions were reported by Kisangau *et al*., (2007*)* where petroleum ether, dichloromethane, and water extracts of *P. barbatus* were reported to inhibit the growth of *Staphylococcus aureus, B. subtilis, E. coli, Pseudomonas aeruginosa.*

Essential oils extracted from *Plectranthus* species have also been reported to possess antimicrobial activity. For example, essential oil of *P. amboinicus* has been reported to possess antifungal activity against *Aspergillus flavus, A.niger, Aspergillus ochraceus, Aspergillus oryzae, Candida versatilis, Fusarium moniliforme* and *Saccharomyces cerevisiae* in stored food products by Murthy *et al*. (2009). Essential oil of *P. amboinicus* has also been reported to inhibit the growth of *C. albicans, Candida tropicalis, Candida guilliermondii* and *Candida krusei* by Oliveira *et al*. (2007). Another study revealed that essential oil of *P. ornatus* has antibacterial activity against *S. aureus, S. pyogenes, E. coli* and *S. typhimurium* (Oliviera *et al*, 2007). Essential oil of *P. montanous* is said to have antifungal activity and has been shown to inhibit the growth of *C. albicans, Microsporum canis, Microsporum gypseum, Trichophyton rubrum, Trichophyton mentagrophytes, Fusarium oxysporum, Curvularia lunata, Stemphyllium solani* (Marwah *et al*., 2007). Essential oils are produced in large amounts in *Plectranthus* genus (Labiatae) and they

mainly consists of monoterpenes, sesquiterpenes and diterpenes (Ascensao *et al.,* 1998). It can be concluded that one of the components of the total leaf extracts of *Plectranthus* species and *P.barbatus* partitions were essential oils which could be responsible for most bioactivities in these species.

A study conducted by Asres *et al*., (2013) showed that *P. montanous* has significant antibacterial activity against *Escherichia coli, Shigella* sp. and *Vibrio cholerae* and antifungal activity against *C. albicans* and *A. niger* and these findings support the current study. Lastly, *P. caninus* has been observed to have low antimicrobial activity (Tadesse *et al*., 2011) and this is also in line with the current study findings where the antimicrobial activity of *P. caninus* was found to be significantly different from that of Amoxicillin. Although various previous studies on the antimicrobial activity of the studied *Plectranthus* species have been done, the antimicrobial activity of *P. pseudomarrubioides, P.edulis, P.aegyptiacus, P. otostegioides* and *P. lanuginosus* have not been reported before however these species have been reported to possess other bioactivities. For example, aqueous extracts of *P. lanuginosus* have been reported to have antioxidant activity (Fale *et al*., 2009) *P. pseudomarrubioides* has been used as an insect repellent (Omolo *et al*., 2004)

With respect to toxicity, *Plectranthus* species have been argued to have low toxicity in both man and animals (Rice *et al*., 2011). For example, *P. barbatus* generally possesses low toxicity and high antibacterial and antioxidant activities (Figueiredo *et al*., 2010). Similarly, ethyl acetate extract from the leaves of *P.barbatus* has low toxicity on peripheral blood mononuclear cells (Kapewangolo *et al*., 2013). Aqueous extract from the leaves of *P. amboinicus* was found to be non toxic to *Artemia salina* (Parra *et al*., 2001). Moreover, 2000 mg/kg of methanolic crude leaf extract from *P. amboinicus* is non-toxic to male and female swiss albino mice and did not cause

change in haematological and biochemical parameters in the mice (Pillai *et al*., 2011). 13-Epi-sclareol is a labdane diterpene isolated from *P. barbatus* and was observed to have no cytotoxic effects to normal cells notably the vero cell line and primary osteoblast cells (Alasbahi and Melzig, 2010). Ethanolic crude extracts from the aerial parts of *P. ornatus* is moderately toxic to *Artemia salina* (Brasileiro, *et al*., 2006). Furlanetto *et al*. (2012) reported that concoctions from *P. ornatus* and *P. amboinicus* do not present any adverse effects to people using them in the management of ailments. Previous results indicated that *P.caninus* exhibited low cytotoxic with high radical scavenging and anti-inflamatory activity (Aswal *et al*., 1984; Tadesse *et al*., 2011). Currently, there are no previous studies on the toxicity of *P. lanuginosus*, *P. edulis*, *P. pseudomarrubioides* and *P.aegyptiacus.* The fact that *P.edulis* is used as food proves that this species does not have adverse effects on cells. Determination of toxicity is a key step in natural product research because it is assumed that the best drug should have low toxicity but should have high activity against its target (Hopkins, 2008) and this makes *Plectranthus* species better candidates for drug development due to their low toxicity.

Bioassay guided fractionation and phytochemical analysis of the DCM leaf crude extracts of *P. barbatus* led to isolation of two new compounds: A and B. Compound A was identified as (S)-4, 4a-dihydro-5, 8-dihydroxy-7-((S)-2-methoxypropyl)-1, 2, 4a -trimethylphenanthrene-3, 6-dione (Table 4.12) while compound B was identified as (S)-4,4a-dihydro-5,8-dihydroxy-7-((R)-2-methoxypropyl)-1,2,4a-trimethylphenanthrene -3, 6-dione (Table 4.13). When the two isolated compounds were tested against the five microbes through broth dilution to determine their MICs, they were both found to inhibit the growth of the test microbes. MIC values for both compounds ranged from 20 mg/ml to 40 mg/ml (Table 4.11). Compound B had slightly lower MIC values compared to Compound A. It has been argued that antimicrobial activity for a pure compound is

significant when MIC <10 µg/mL, moderate when 10 < MIC < 100 µg/mL and negligible or low when MIC > 100 µg/mL (Kuete, 2010). All the MIC values obtained from the two compounds were above 100 µg/ml and hence the two compounds were considered to have low or negligible antimicrobial activity against MRSA, *B.cereus, E.coli, C.albicans* and *A. niger.*

Compound A and B are abietane diterpenes and this group of diterpenes has been reported to have myriad of bioactivities including antimicrobial activities. For example, ten abietanes diterpenes have been isolated from the acetonic extract of *Plectranthus grandidentatus* Gürke*, Plectranthus hereroensis* and *P. ornatus* which were reported to have antibacterial activity against methicillin resistant *staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus faecalis* (VREF) by Gasper-Marques *et al*. (2006). Another study reported that two abietane diterpenoids isolated from *Plectranthus elegans* have antifungal activity against *Cladosporium cucumerinum* (Dellar, 1996). Two abietane diterpenes known as 7-epigrandidone D and grandidone D from *Plectranthus grandidentatus* have also been reported to have antimicrobial activity (Teixeira *et al*., 1997). Similarly, two abietane diterpenes known as parvifloron D and parvifloron F with antimicrobial activity against *Listeria monocytogenes* and *Mycobacterium tuberculosis* have been isolated from *Plectranthus ecklonii* (Nyila *et al*., 2009). In another study, seven abietane diterpenes isolated from *P. hadiensis, P. ecklonii, P. purpuratus* and *P. lucidus* were reported to have antimalarial activity (Van Zyl *et al*., 2008). Moreover, five known abietane diterpenes isolated from *Plectranthus grandidentatous* were found to have antiproliferative activity against human cancer cell lines (Marques *et al*., 2002). Other abietane diterpenes with reported bioactivities are Colen E from *P. amboinicus* with anticancer activity (Yuilanto *et al*., 2016), Coleon E from *P. barbatus* with antioxidant activity (Porfirio *et al*., 2010) and (16S)-Coleon E with antiacetylcholinesterase and antioxidant activity (Fale' *et al*., 2009). In summary,

Plecranthus genus is rich in diterpenes and many species of *Plectranthus* produce diterpenoids which include kaurane, phyllocladanes, beyeranes, primanes and abietane diterpenes which have promising bioactivities (Grayer *et al.*, 2010).

By comparing growth inhibitions of the DCM: MeOH leaf crude extracts among the ten *Plectranthus* species, generally high growth inhibitions were observed in *P.barbatus*, *P.lanuginosus*, *P.otostegioides* and *P.ornatus*. From the phylogenies inferred from the leaf micromorphology, molecular data and those from previous studies, *P.barbatus, P. lanuginosus*, *P.caninus* and *P.otostegioides* have been shown to be closely related. In addition, the results from the leaf micromorphology and molecular phylogeny study also indicated that *P. montanous, P. amboinicus, P. pseudomarrubioides* and *P. aegyptiacus* are closely related to each other. Hence there is a correlation between taxonomic grouping of *P. barbatus, P. lanuginosus* and *P. otostegioides* and their antimicrobial activity since these three species inhibited the growth of microbes in a similar manner and are closely related. It can be concluded that close genetic relationship between these three species could imply that they share a biosynthetic pathway for producing similar phytoconstituents which are responsible for the observed similarity in antimicrobial activity.

Apart from being present in *P. barbatus*, compound A is also present in *P. otostegioides*, *P. ornatus* and *P. caninus* (Figure 4.31). From the molecular phylogeny and leaf micromorphology results. *P. barbatus*, *P. otostegioides* and *P. caninus* have been grouped together. As a result presence of compound A in *P. barbatus*, *P. otostegioides* and *P. caninus* has a chemotaxonomic significance. *P. barbatus*, *P. otostegioides* and *P. caninus* can produce compound A implying that they are closely related and this close relationship has been supported by the molecular phylogeny and leaf micromorphology results. However compound B was found in species which are not

grouped together from the molecular phylogeny and leaf micromorphology data which include: *P. barbatus, P. amboinicus, P. montanous, P. ornatus* and *P. caninus* (Figure 4.32). Results of the phylogenetic studies showed that *P. amboinicus* is closely related to *P. montanous* while *P. barbatus* is closely related to *P. ornatus* and *P. caninus.* The fact that compound B is found in the four species which do not share a more recent common ancestor implies that compound B does not have any chemotaxonomic significance as far as the four species which produce compound B are concerned.

5.2. Conclusions

From the results, a phylogenetic tree reconstructed using *MatK* genes grouped *P. caninus*, *P. otostegioides, P. barbatus* and *P. lanuginosus* together (Cluster A). Again based on the *MatK* genes; *P. pseudomarrubioides*, *P. ornatus*, *P. montanous* and *P. amboinicus* were shown to be closely related and hence grouped together in Clusters B and C. Still based on *MatK* phylogenetic tree, the aforementioned species were shown to share a distant common ancestor with *P.edulis*. The same trend was observed in the phylogenetic tree created using the *Rbcl* genes sequences (Cluster D and E) although *Rbcl* phylogenetic tree grouped *P.edulis* close to *P.amboinicus* and showed *P. aegyptiacus* to be distantly related to the other nine species despite being grouped close to *P. pseudomarrubioides* and *P.ornatus* by the *MatK* phylogenetic tree.Thus the phylogenetic trees based on rbcl and matk have potential to deifferentiat the problematic species of *Plectranthus*.

From the leaf micromorphology study, *P. caninus, P.ornatus, Potostegioides, P.montanous* and *P. pseudomarrubioides* are closely related (Cluster F) while *P. aegyptiacus, P. amboinicus, P. edulis, P. barbatus* and *P. lanuginosus* are closely related as well (Cluster G). Furthermore, the leaf micromorphology study showed that *P. caninus* is closely related to *P. ornatus* while *P. otostegioides*, *P. montanous* and *P. pseudomarrubioides* have been shown to be related as well. *P.*

aegyptiacus has been placed close to *P. ambonicus* and lastly *P. edulis, P. barbatus* and *P. lanuginosus* have been grouped together as well. The findings from the study have been supported by findings from previous morphological, biochemical and molecular studies which have reported that *Plectranthus* species are closely related. It can be concluded that, studies of leaf micromorphology have potential in providing diagnostic morphological characters which can aid in plant identification and phylogenetic studies.

Analysis of morphological data through cluster analysis clusters species together based on their overall similarity and this grouping can be presented in form of cladograms, dendrograms or even phylogenetic trees. Morphological characters have various shortcomings especially when used to delimit species at the genus level. Most of these characters overlap and it is hard to identify the diagnostic characters which can be used to differentiate one species from the rest. It has been argued that, the most suitable method for classifying species based on morphological data has not yet been decided and different methods applied on the same data can result to different classifications. However, analysis of morphological data can prove valuable when placing a new species within a higher taxonomic rank (Wiley, 1981).

Although molecular and biochemical data has many advantages and is increasingly being used in systematic studies, it is crucial to note that morphological systematics should not be abandoned because it can aid in the classification of poorly known groups as well as study of fossil data (Hillis and Wiens, 2000). The first process of identifying a collected plant species is describing it by listing its morphological characters. Hence, if plant taxonomists focus entirely on molecular and biochemical techniques, then they might not to be able to identify new species and research on unidentified species may come to a halt. Correct identification of species in the field and in the herbarium is based on the species morphological data (Maddison, 1996).

With respect to antimicrobial activity, DCM: MeOH crude extracts of *P. barbatus*, *P. lanuginosus* and *P.ornatus* have been reported to have antimicrobial activity. 200 mg/ml of DCM: MeoH (1:1) leaf crude extracts of *P. barbatus* and *P. lanuginosus* had antibacterial activity against MRSA and the activity was not significantly different from the antibacterial activity of 50mg/ml Amoxicillin against MRSA. Again 200 mg/ml of *P.ornatus* and *P. barbatus* had antifungal activity against *C.albicans* which was not significantly different form that of 40mg/ml of Ketoconazole. The study also reports for the first time, the antimicrobial activity of *P. pseudomarrubioides, P.edulis, P. aegyptiacus, P. otostegioides and P. lanuginosus*. DCM: MeOH crude extracts of *P.barbatus* displayed the lowest MIC levels ranging from 25 mg/ml to 100 mg/ml against *MRSA, B.cereus, E.coli, C.albicans* and *A.niger* compared to the total leaf extracts of the other nine *Plectranthus* species. Further antimicrobial activity of *P. barbatus* was observed in the DCM partition of *P.barbatus* leaves which also had lowest MIC levels ranging from 40 mg/ml to 150 mg/ml against the microbes investigated compared to the other partitions of *P.barbatus*.

From the crude DCM extracts of *P.barbatus,* two new abietane diterpenes were isolated using Liquid Column Chromatography and identified by spectroscopic means. Compound A was identified as (S) - 4, 4 a – dihydro - 5, 8 – dihydroxy – 7 - ((S) – 2 - methoxypropyl) - 1, 2, 4 a – trimethylphenanthrene -3, 6 - dione while compound B was identified as (S)-4, 4 a – dihydro - 5, 8-dihydroxy - 7 - ((R) – 2 - methoxypropyl) -1, 2, 4a - trimethylphenanthrene -3, 6-dione. These two compounds were simply referred to as (16S)-MethoxyColeon E i.e Compound A and (16R)-MethoxyColeon E i.e Compound B due to their similarity to Coleon E. Their antimicrobial activity against the test microbes was considered low or negligible because their MIC values were above 100 µg/ml which ranged from 20 mg/ml to 50 mg/ml.

5.3. Recommendations

Current study indicates that further research involving more *Plectranthus* species within the *Coleus* and *Plectranthus* clade is needed to better understand *Plectranthus* taxonomic relationships. Roughly one hundred *Plectranthus* are found in East Africa; about seventy of the species belong to the *Coleus* clade and about thirty of them belong to the *Plectranthus* clade (Paton *et al*., 2009). Investigations on molecular phylogeny of all the species will help researchers in dealing with the problems of synonymy and classify *Plectranthus* species correctly. Such studies will not only involve the use of *MatK* and *Rbcl* genes but it can also involve the use of other molecular markers and methods which have been used sussessfully in phylogenetic studies of angiosperms such as *ndh-F, atpB, trnT–trnL, trnL–trnF* and *atpB-Rbcl.* A comparison of phylogenetic trees based on two or three gene sequences can prove valuable in systematics.

Morphological characters are affected by environmental factors and the same morphological character within the same species can be pronounced or decreased depending on the ecological zone where the species is thriving. Hence it is very important for one to establish whether differences among species observed from morphological characters are due to actual genetical differences or environmental factors. Species classification using morphological data is usually based on diagnostic characters rather than phylogenetic analysis and the classification produced may not give the correct evolutionary relationships of the species involved. For one to make accurate phylogenetic inferences from morphological data dendrogram, it is important to compare such dendrograms with phylogenetic trees based on molecular or biochemical data.

Many species within *Plectranthus* genus have been used in the management of many diseases and several studies have confirmed that these species have antimicrobial activities. Studies on *Plectranthus* species have also led to the isolation of compounds from these species

and screening of the isolated compounds for bioactivities has been done. However, *Plectranthus* species are rich in saponins, monoterpenes, sesquiterpens, diterpenes and phenolic compounds and more research geared towards isolation of these compounds from *Plectranthus* species coupled with bioassays should continue. This could lead to the isolation of more compounds with higher bioactivities. Wide usage of *Plectranthus* species in ethnomedicine notably *P.barbatus* and its documented high antimicrobial activities and low toxicity proves that this plant can be vital as a source of other bioactive novel products or chemical templates due to its ability to inhibit growth of pathogenic microbes. Besides, research on the distribution of phytoconstituents in *Plectranthus* species can help in the classification of the genus and compliment classification based on molecular and morphological characters.

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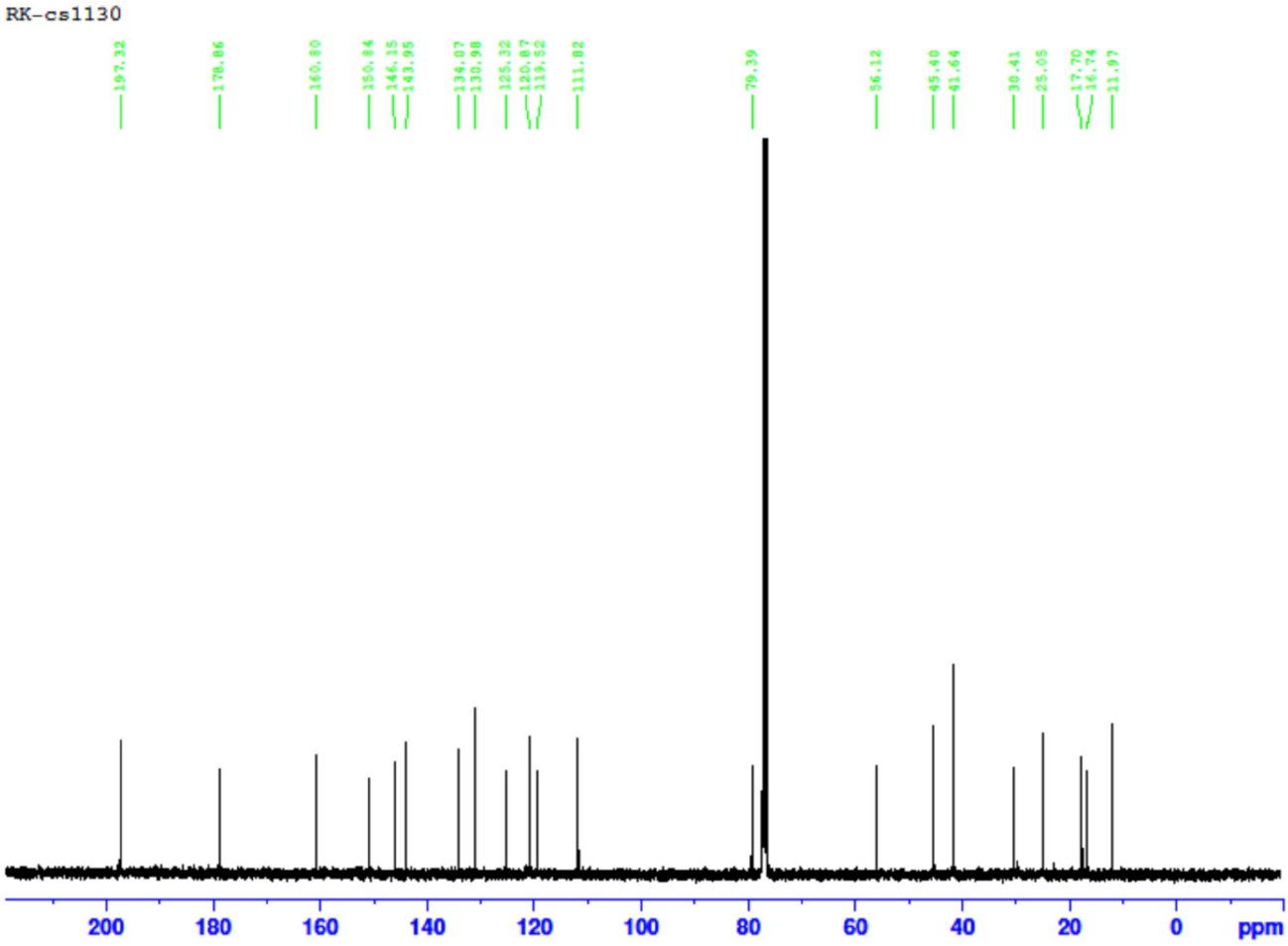
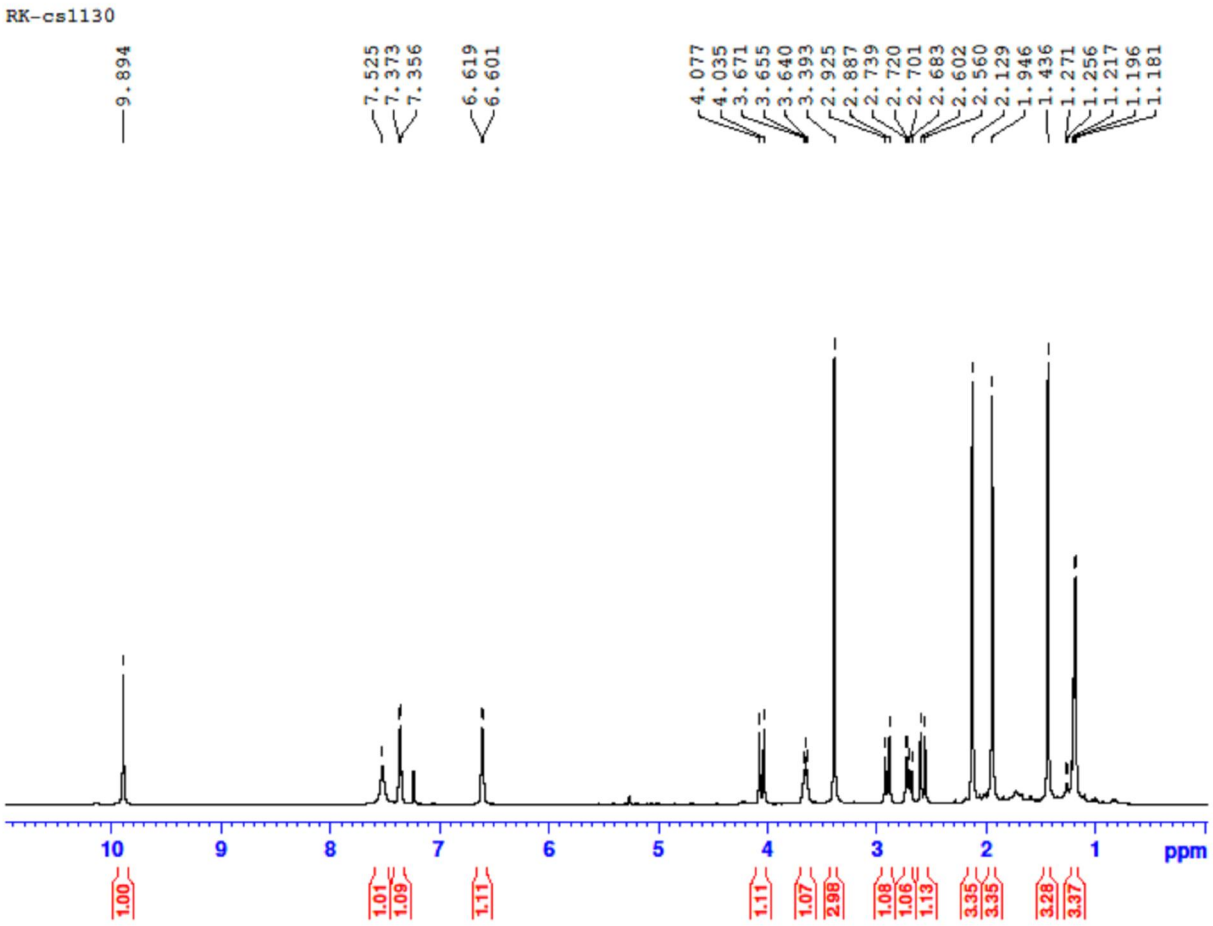
APPENDICES

Appendix 1: 1D NMR, 2D NMR and MS for Compound A

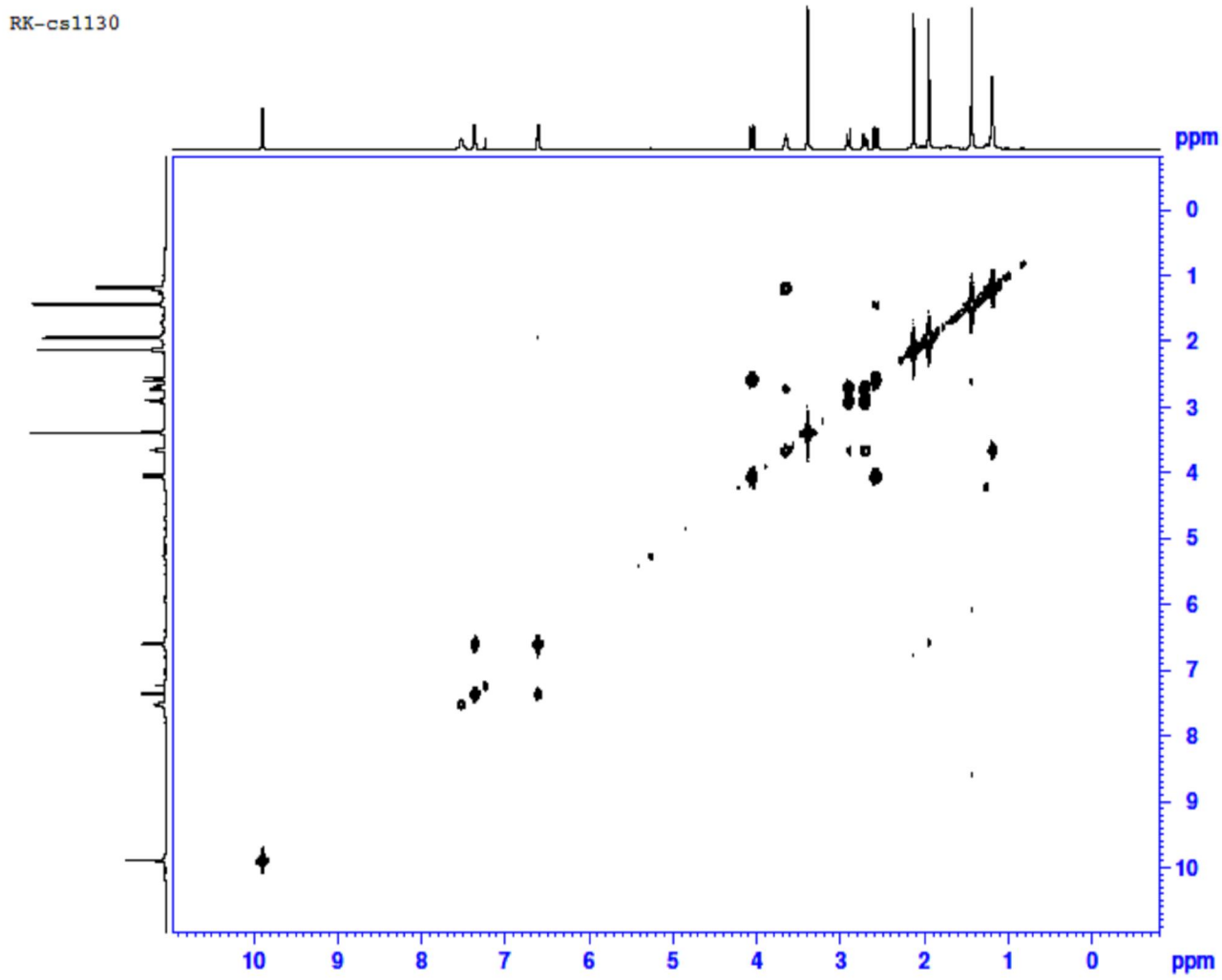
1.1. ^{13}C NMR in $CDCL_3$ at 400 MHz for Compound A



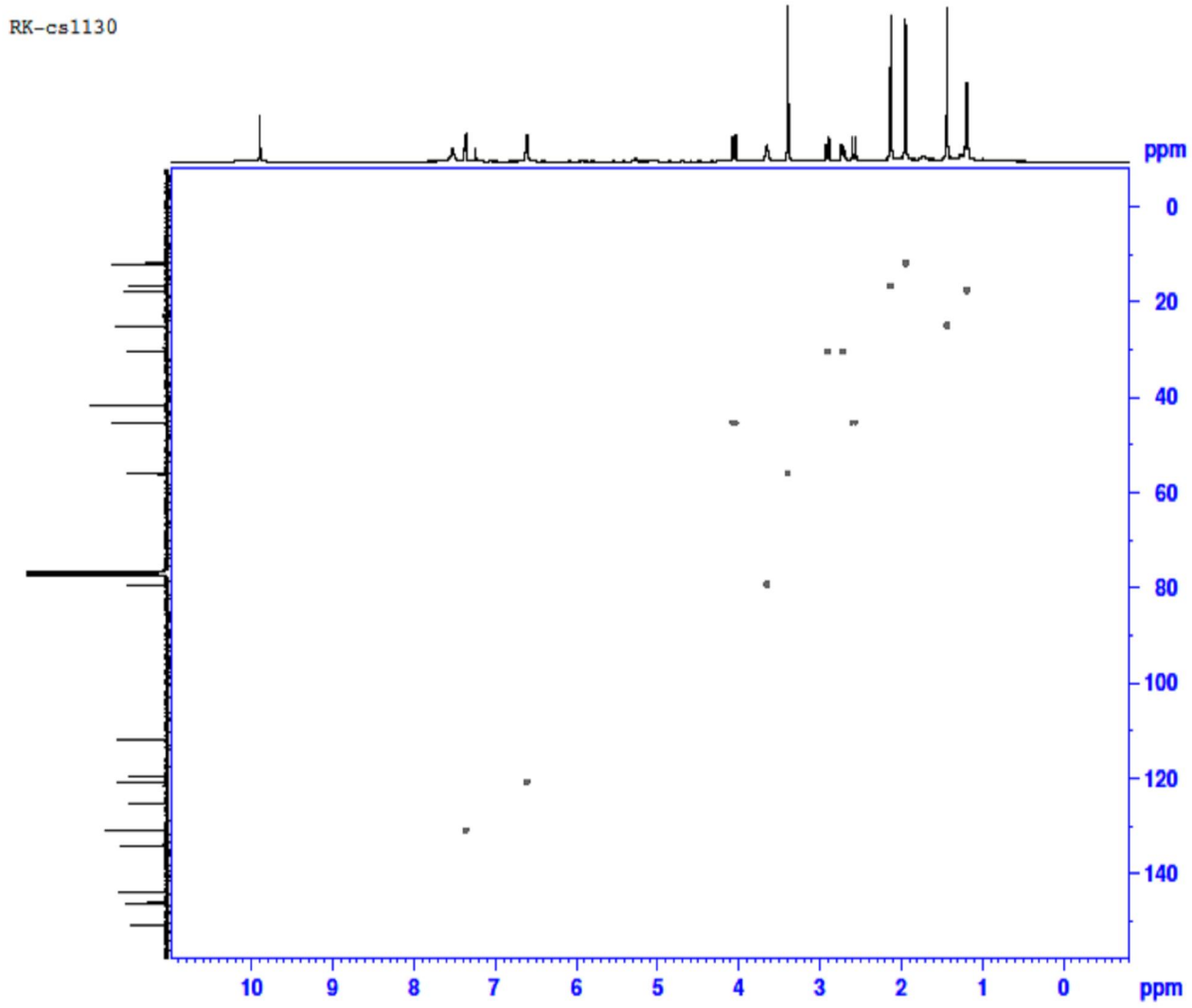
1.2. ^{1}H NMR in $CDCL_3$ at 400 MHz for Compound A



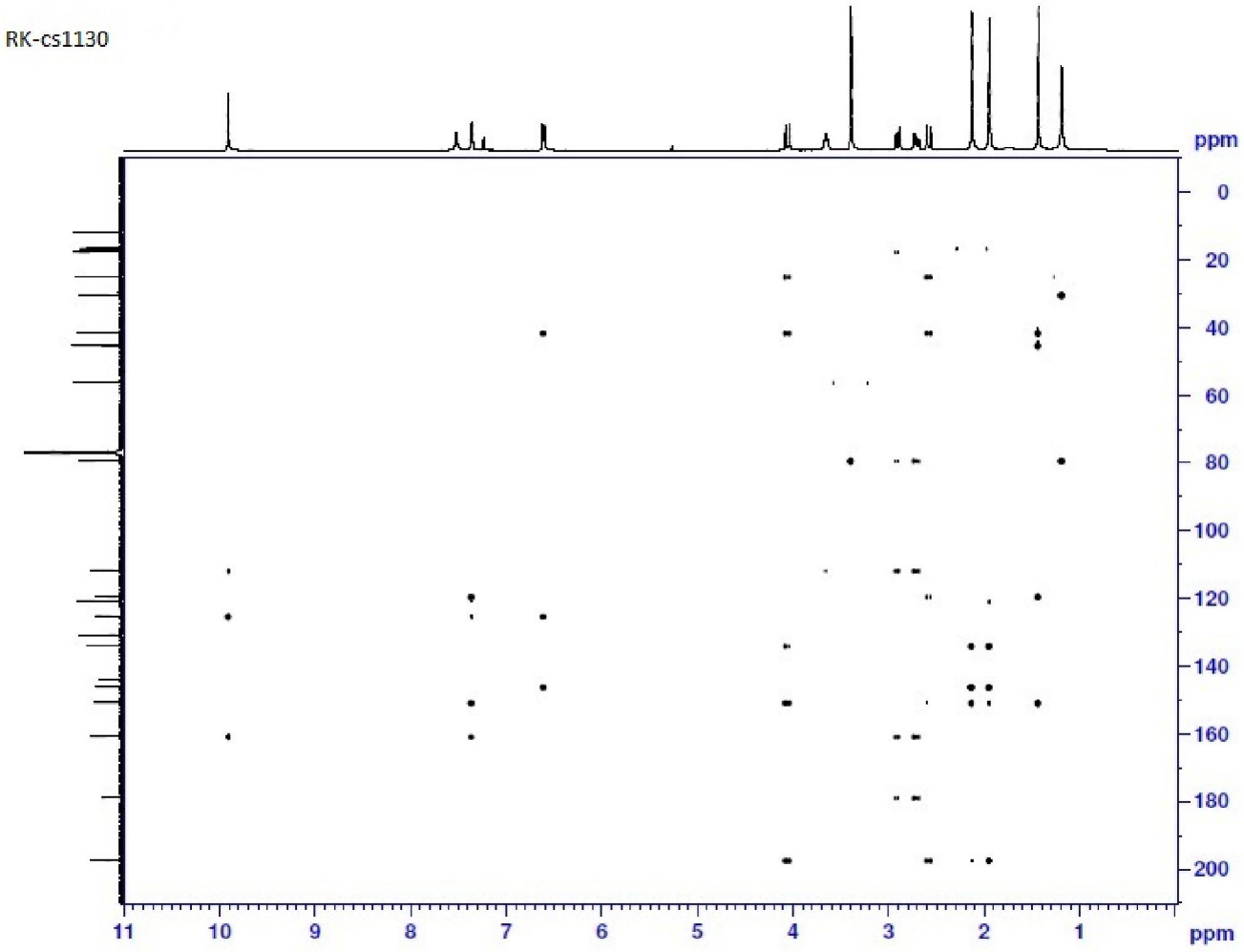
1.3. Homonuclear Correlation Spectroscopy (COSY) for compound A



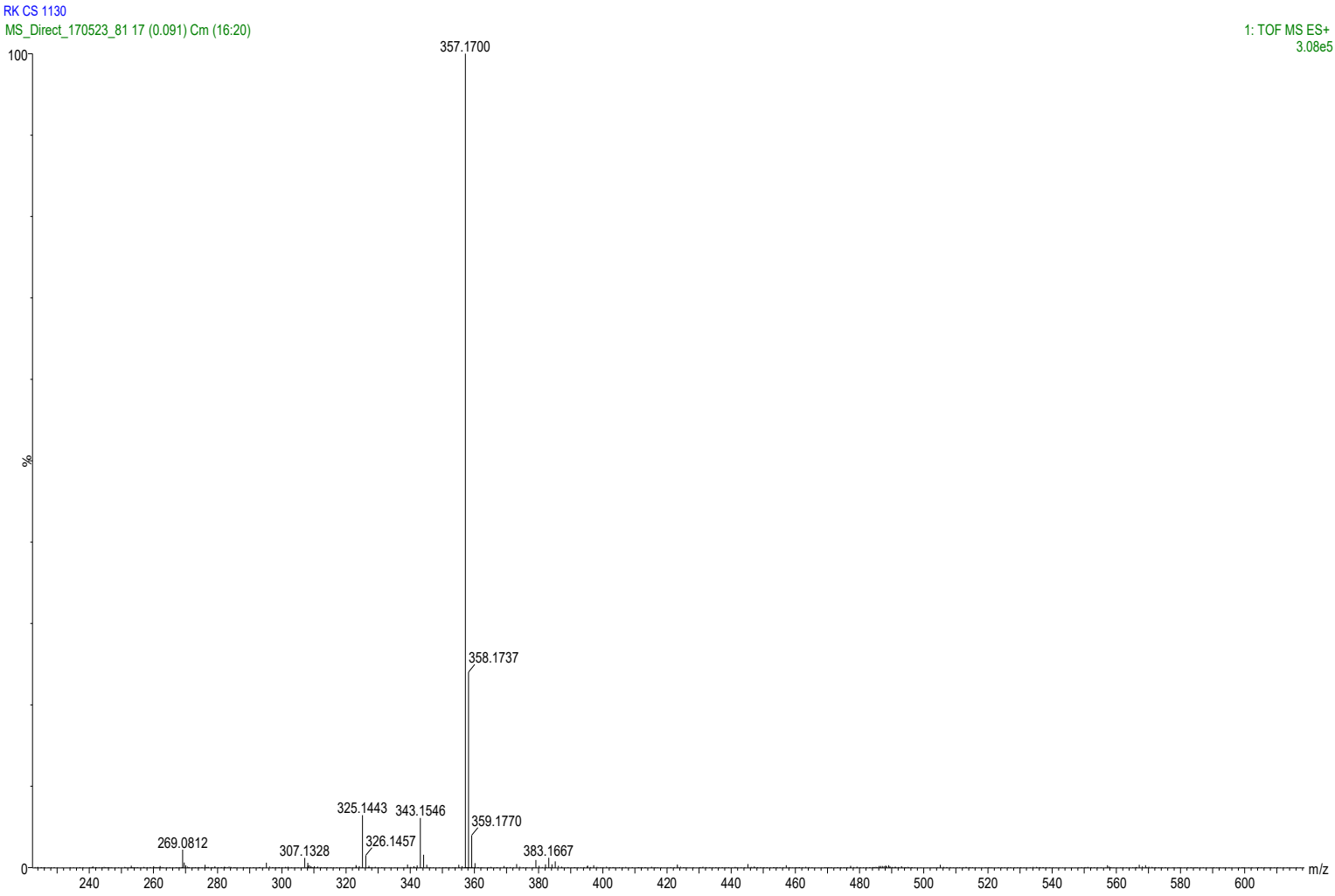
1.4. Heteronuclear Single-Quantum Correlation (HSQC) for Compound A



1.5. Heteronuclear Multiple Bond Correlation (HMBC) for Compound A

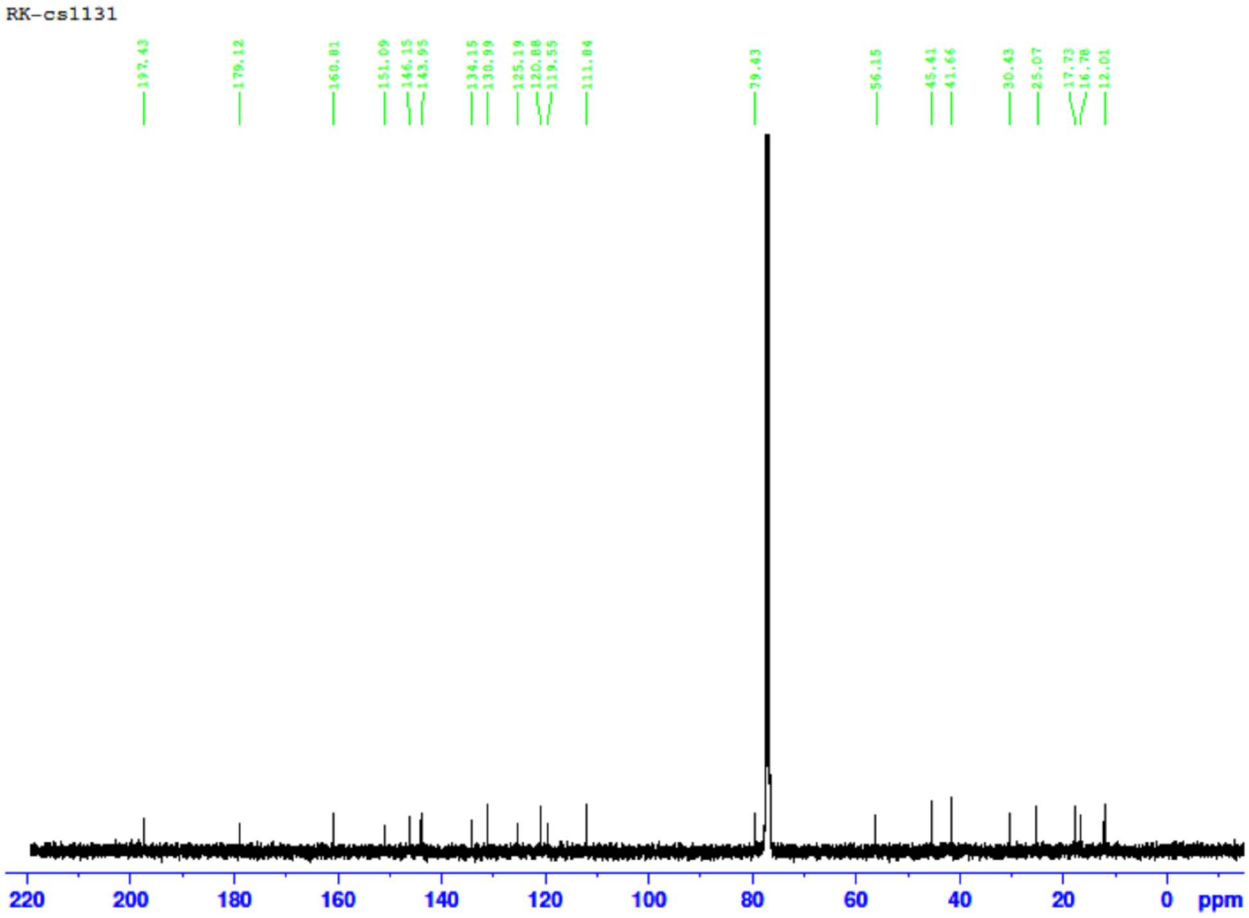


1.6. ESTOF High resolution Mass Spectrum for Compound A

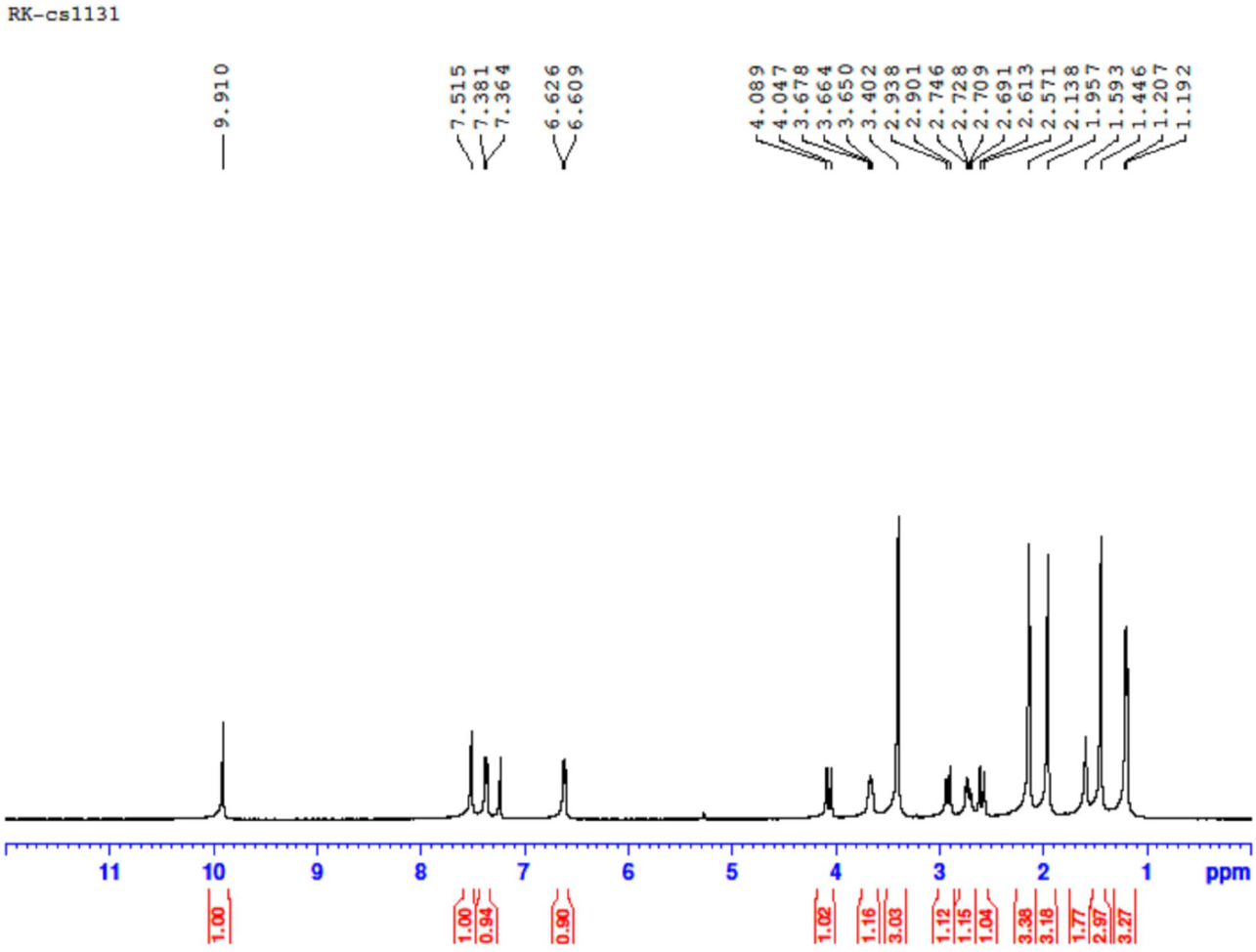


Appendix 2. 1D NMR, 2D NMR and MS for Compound B

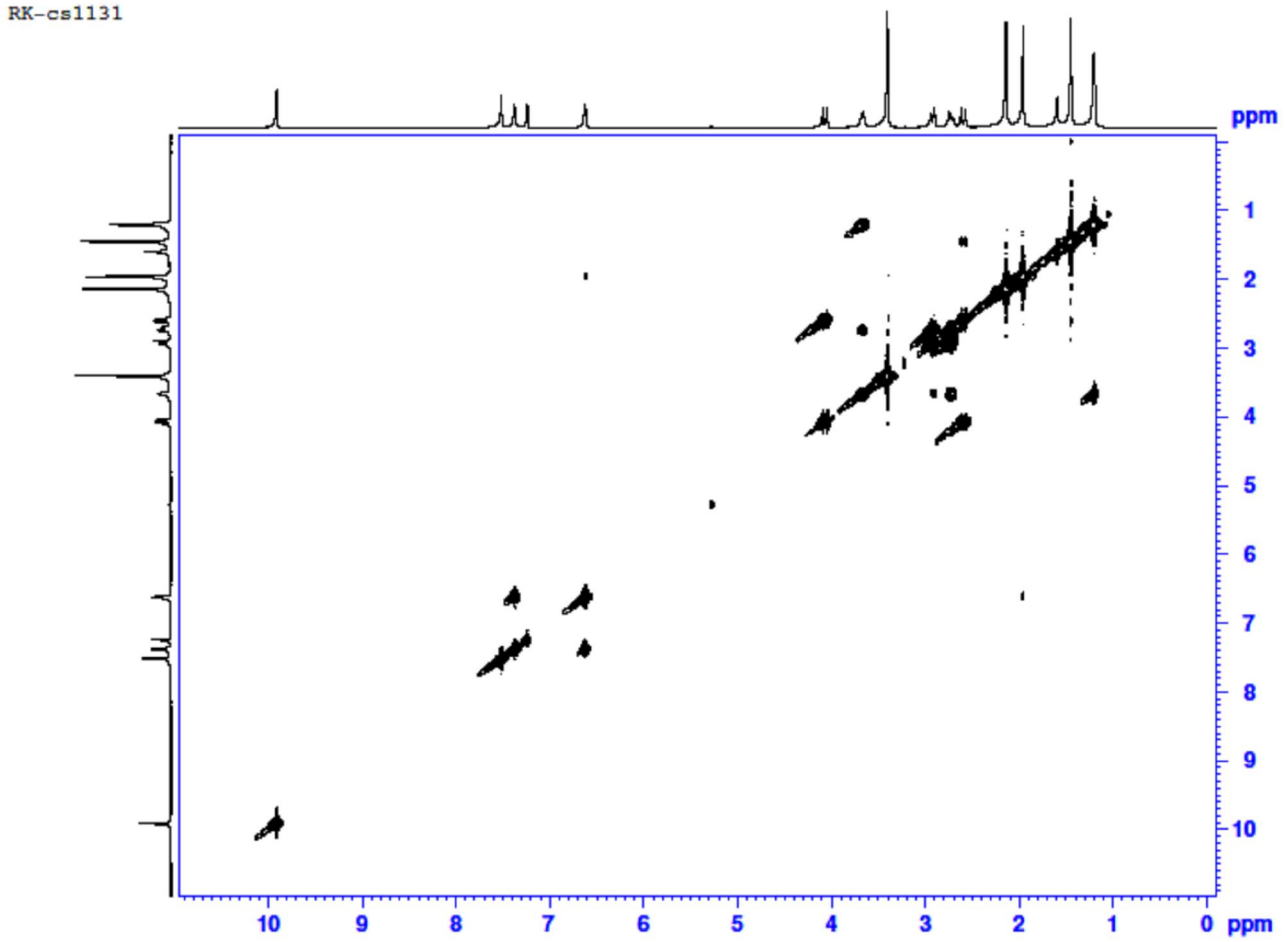
2.1. ^{13}C NMR in $CDCL_3$ at 400 MHz for Compound B



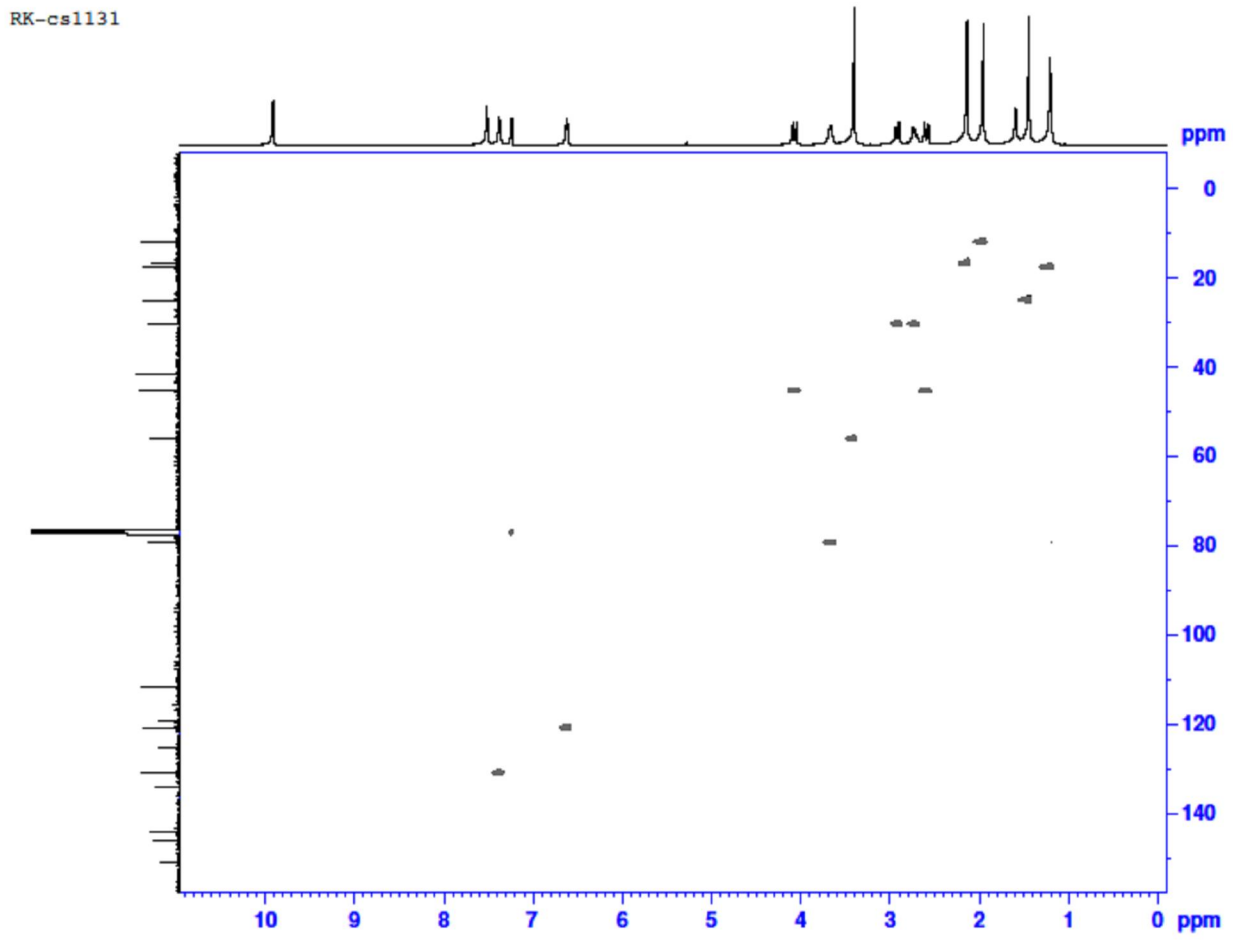
2.2. ^{1}H NMR in $CDCL_3$ at 400 MHz for Compound B



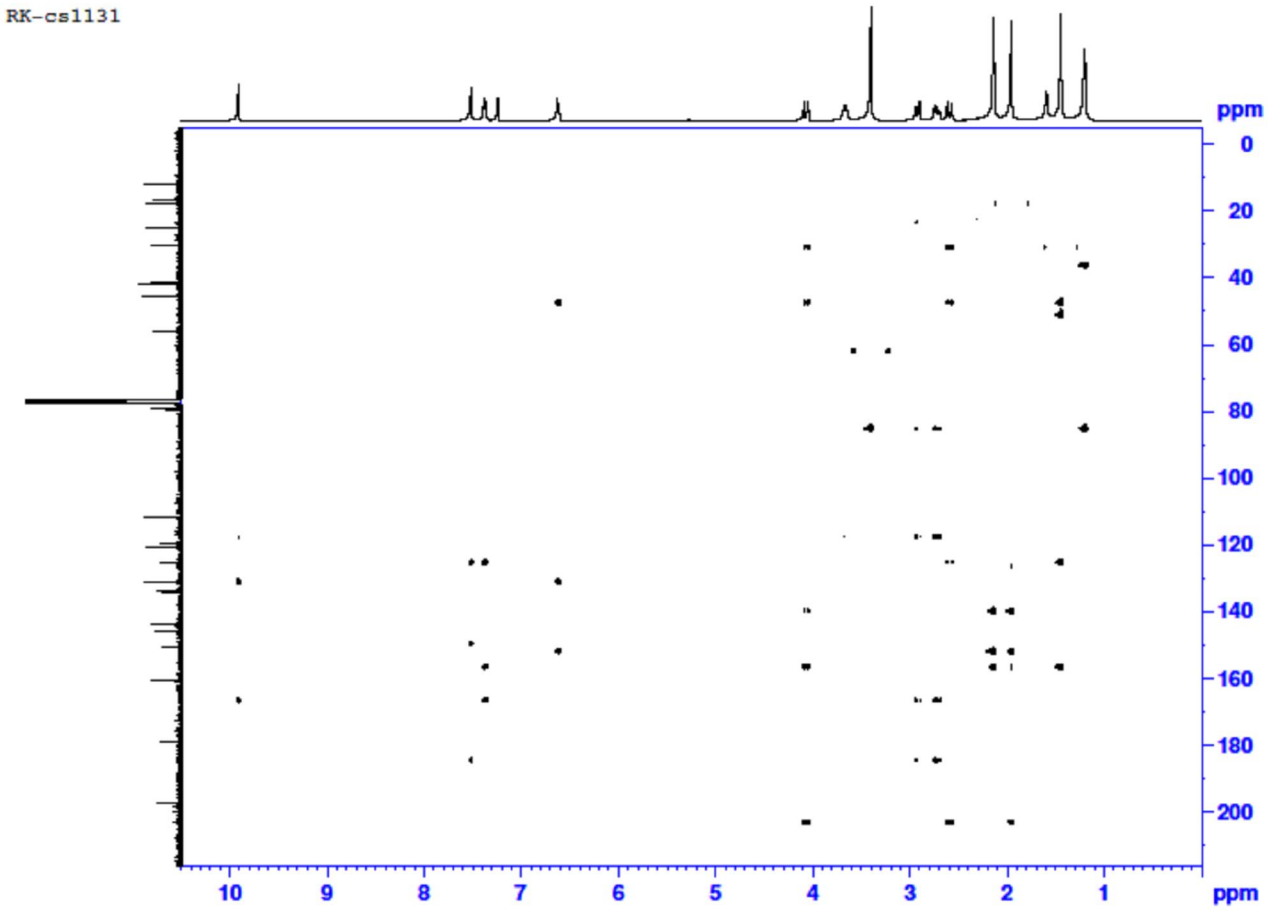
2.3. Homonuclear Correlation Spectroscopy (COSY) for compound B



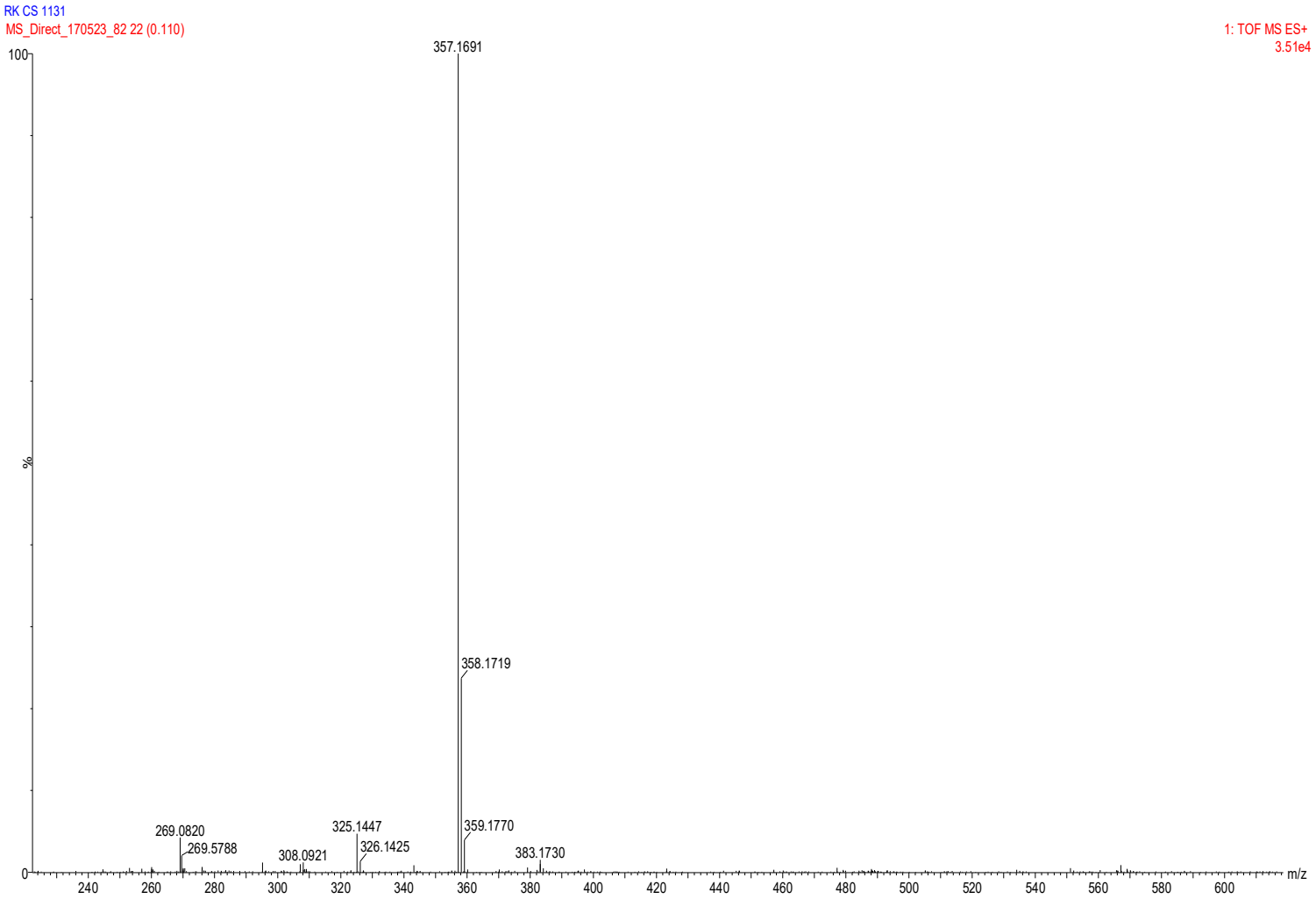
2.4. Heteronuclear Single-Quantum Correlation (HSQC) for Compound B



2.5. Heteronuclear Multiple Bond Correlation (HMBC) for Compound B



2.6. ESTOF High resolution Mass spectrum for Compound B



PUBLICATIONS

Musila, F M, Nguta, J. M, Lukhoba, C. W and Dossaji, S. F. (2017). Antibacterial and antifungal activities of ten Kenyan *Plectranthus* species in the *Coleus* clade, *Journal of Pharmacy Research*, 11(8), 1003-1014.(Published August 2017)

Musila, F. M, Lukhoba, C. W, Nguta, J. M and Dossaji, S. F. (2017). Phylogeny of ten Kenyan *Plectranthus* species in the *Coleus* clade inferred from leaf micromorphology, *Rbcl* and *MatK* genes, *Journal of Botany*. (Published December 2017).

Musila, F.M, Lukhoba, C. W., Nguta, J. M and Dossaji, S.F. (2017). Molecular Phylogeny of ten Kenyan *Plectranthus* species in the *Coleus* clade, *In proceedings of XXI AETFAT congress*. Catholic University of Eastern Africa, Nairobi, Kenya (Presented May 2017).

Musila, F. M, Dossaji, S. F., Lukhoba, C. W and Nguta, J. M (n.d).Two new abietane diterpenes with antimicrobial activity from *Plectranthus barbatus* (Unpublished)