

ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF CRUDE EXTRACTS
AND FRACTIONS FROM THE STEMS AND LEAVES OF *TEPHROSIA*
LUPINIFOLIA

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ABSTRACT

Tephrosia lupinifolia is used traditionally for the treatment of malaria, diarrhea, toothache and tuberculosis. Two flavonoids, lupinifolin and lupinifolinol were previously isolated from the hexane root extract of *T. lupinifolia*, but there are no reports on phytochemical and pharmacological studies conducted on the stems and leaves of the plant. The purpose of this study was to prepare and fractionate crude extracts from the leaves and stems of *T. lupinifolia*, and to evaluate their antioxidant as well as antimicrobial activities. Crude extracts were prepared by suspending the powdered plant material in an equivolume mixture of dichloromethane and methanol at room temperature for 48 hours on an orbital shaker. The extracts were then subjected to a combination of trituration, column chromatography and preparative TLC, which yielded nine partially purified fractions as revealed by Gas Chromatography-Mass Spectrometry (GC-MS) analysis. The fractions and crude extracts were tested for antimicrobial activity against one gram-positive bacterium (*Staphylococcus aureus*), two gram-negative bacteria (*Klebsiella pneumonia* and *Escherichia coli*) and one fungal strain (*Candida albicans*), using the agar disc-diffusion method. The best activity, albeit moderate, was recorded for the crude stem extract with zones of inhibition of 14 and 12 mm against *K. pneumonia* and *C. albicans*, respectively. The crude leaf extract displayed equipotent activity against *E. coli* and *C. albicans* with zones of inhibition of 10 mm. According to the GC-MS analysis fraction L5, which was obtained by subjecting the crude leaf extract to column chromatography, consisted of 10 compounds and the major compound was tentatively identified as 4-tetradecane. Fraction L6 comprised of four compounds of which two major compounds were tentatively identified as hexadecane and cyclohexanone with the aid of mass spectral libraries. Only fractions S1, S2, S3, L1,

L5 and L6 were tested for antioxidant activity using the 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) assay. The test revealed that S3 showed the highest IC_{50} value of 23.50 ± 3.28 mg/mL whereas the lowest, and thus the best, antioxidant activity was recorded for fraction L6 with an IC_{50} of 0.80 ± 0.01 mg/mL.

Quantitative determination of the total phenolic and total flavonoid contents of the methanolic leaf, root and stem extracts was done using the Folin Ciocalteu method and aluminum chloride complex forming assays, with the results expressed in mg of gallic acid equivalents (GAE)/g of dry weight (DW) and mg of quercetin equivalents (QE)/g DW, respectively. Total phenolic content was recorded as 45.3 mg GAE/g DW for the leaves, 58.3 mg GAE/g DW for the roots and 250.5 mg GAE/g DW for the stems. Flavonoid contents were obtained as 31.8 ± 0.032 mg QE/g DW for the leaves, 13.2 ± 0.003 mg of QE/g DW for the roots and 20.3 ± 0.013 mg of QE/g DW for the stems. The methanolic stems extract showed the highest total phenolic content whereas the highest total flavonoid content was shown from the methanol leaves extract.

Keywords: Antimicrobial activity, antioxidant activity, ethnomedicine, phytochemical screening, *Tephrosia lupinifolia*.

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LIST OF ABBREVIATIONS

AR	Analytical reagent
CC	Column Chromatography
CHCl₃	Chloroform
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DPPH	α, α -Diphenyl- β -picrylhydrazyl
EA	Ethyl acetate
g	Grams
GC	Gas chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GPS	Global positioning satellites
GAE	Gallic acid equivalent
H₂SO₄	Sulphuric acid
HCl	Hydrochloric acid
HPLC	High-Performance Liquid Chromatography
IC₅₀	Half-maximal inhibitory concentration
IR	Infrared
KOH	Potassium hydroxide
MeOH	Methanol
MIC	Minimum inhibitory concentration
mL	milliliter
MS	Mass Spectrometry
MET	Ministry of Environment and Tourism
Na₂CO₃	Sodium carbonate

NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBRI	National Botanical Research Institute
¹H-NMR	Proton Nuclear Magnetic Resonance
R_f	Retention factor
SEM	Standard error of the mean
TPC	Total phenolic content
TFC	Total flavonoid content
TLC	Thin Layer Chromatography
UHPLC	Ultra High Performance Liquid Chromatography
UV	Ultraviolet
QE	Quercetin equivalent

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DEDICATION

This work is dedicated to my family: my late grandmother, Noah Martha Lipitwa, you are gone, but I still remember you and count on you for everything, may your soul continue to rest in everlasting peace. It also goes to my mother, Tresiah Nghiningishiwa, my father, Nanhapo Festus, brothers (Elia, Daniel and Filemoni), sisters (Fiina and Helaria) and lastly it goes to Mrs. and Mr. Nekongo for their great input and for everything they invest into my life as well as to the entire family. May the power of God be with you!

DECLARATION

I, David Nanhapo, hereby declare that this study is my own work and is a true reflection of my research and that this work or any part thereof has not been submitted for a degree at any other institution.

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Name of Student	Signature	Date

1. CHAPTER ONE: INTRODUCTION

1.1 Orientation of the study

Traditionally used medicinal plants continue to serve as an affordable and accessible treatment for various ailments [1]. The confirmation of the safety and efficacy of these herbal remedies by using scientific methodologies as well as biochemical testing protocols, will not only facilitate their integration into the primary health care systems but also contribute towards the preservation of indigenous knowledge [2].

Natural products or secondary metabolites sourced from ethnomedicinally used plant species, serve as a reliable source of therapeutic agents against infectious and non-infectious diseases [1]. These structurally diverse lead compounds with drug-like properties can be used as templates for the design and synthesis of analogs which, at times, display similar or improved biological activities compared to the parent natural product. For example, lupinifolin, a flavonoid isolated from the hexane extract of the roots of *T. lupinifolia*, was used as a template in the design and synthesis of anticancer metal complexes. The complexes reportedly displayed superior activity compared to lupinifolin [2, 3]. The development of new extraction technologies has modernized the isolation of natural products and aided the discovery of new drugs [4].

Medicinal plants also played an important role in the discovery of antimicrobial and antioxidant agents. Antimicrobial drugs are used in the treatment and/or prevention of microbial infection. They exert their mechanism of action, by killing or inhibiting the growth of microorganisms [5]. Antioxidants are defined as substances which can scavenge free radicals in the human body. Various degenerative diseases such as cancer, cardiovascular diseases, neurological disorders and diabetes are caused by

free radicals in the human body [6, 7]. The use of herbal remedies containing antioxidants has gained importance due to their potential health benefits and their ability to down-regulate degenerative processes [8]. Isolated from *Tephrosia purpurea* are tannins, flavonoids and triterpenoid glycoside which are among the main chemical compounds synthesized by plants even though they are not considered as essential for plant growth, they exhibit several pharmacological properties such as anti-inflammatory, antiulcer, hepatoprotective, antidiarrhoeal, anthelmintic, alexeteric, antipyretic, antibacterial, antimicrobial, antihyperglycemic, immunomodulatory and antiallergic activity [5].

Tephrosia lupinifolia grows in open grassy places, often on sandy soils and is native to African countries such as Angola, Botswana, Namibia, Congo, Senegal, northern Nigeria, Malawi, Zambia, Zimbabwe and South Africa [9, 10, 11]. In Namibia, *T. lupinifolia* is distributed in the central and northern regions as shown in Figure 1 [9, 11].

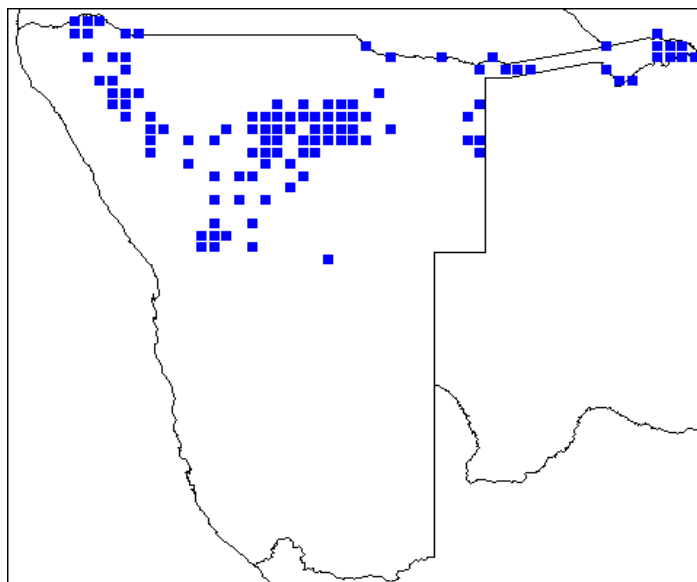


Figure 1: Geographical distribution of *T. lupinifolia* in Namibia [11]

It is a perennial herb with prostrated branches of up to 1m long that grows from a vertical woody rootstock. The stem is covered with spreading hair and it has palmately divided leaves with pink to purple flowers [10]. *T. lupinifolia* is known in Namibia by the vernacular names: *kàq'm* (Khoisan), *Okanakafukwa* (Oshiwambo) and *Platertjie* (Afrikaans) [12].

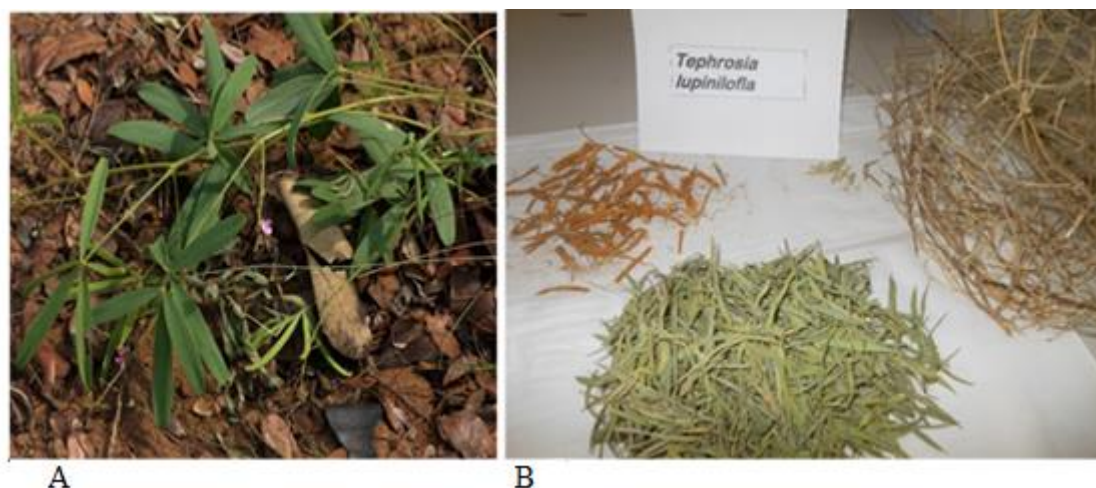


Figure 2: *T. lupinifolia*: A - in its natural state [10] and B - dried plant materials.

The roots of *T. lupinifolia* are traditionally used by indigenous people as an herbal medicine for the treatment of diseases such as malaria, diarrhea, tuberculosis and toothache [13]. Smalberger *et al* [14], reported the isolation of prenylated lupinifolin and lupinifolinol (figure 3) from the hexane root extract of *T. lupinifolia*. The two prenylated flavonoids were tested for antimicrobial activity using the agar disc-diffusion method and they both displayed promising growth inhibitory activity (with a zone of inhibition ≥ 14 mm) against gram-positive (*Bacillus cereus*) and gram-negative (*E. coli*) bacteria [14].

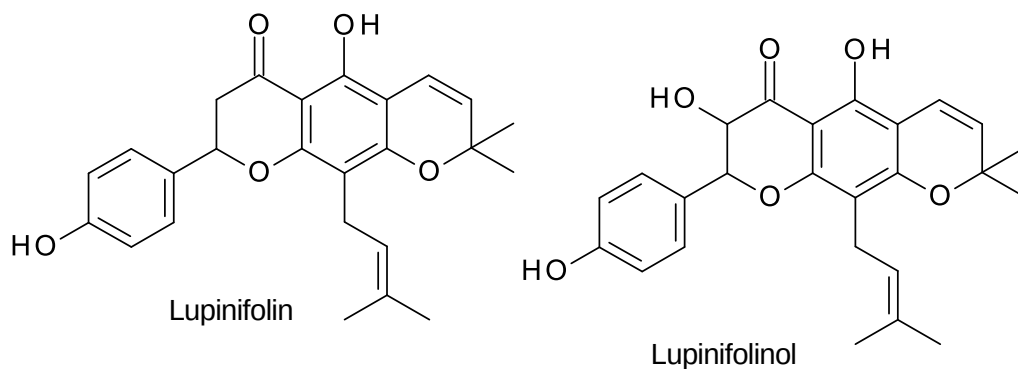


Figure 3: Chemical structures of two flavonoids isolated from *T. lupinifolia*.

A comparison of the antimicrobial activity of the ethanolic root extract of *T. lupinifolia* with lupinifolin showed that the ethanolic root extract displayed better activity against *Corynebacterium diphtheria* and *Bacillus cereus* compared to lupinifolin. Lupinifolin also showed selective cytotoxicity towards breast cancer cell lines [15, 16, 17]. The ethanolic root extract and lupinifolin were also tested for antioxidant activity, using DPPH assay and both showed strong activity in the scavenging of free radicals [3].

1.2 Statement of the problem

As mentioned earlier, phytochemical analysis of the root extract of the South African *T. lupinifolia*, yielded two flavonoid derivatives [18]. Previous studies on the phytochemical screening of biochemical compounds on other species from the genus *Tephrosia* revealed the presence of flavonoids, chalcones, tannins, alkaloids, saponins and phenolic compounds, which represent phytochemical classes which are antimicrobial, anti-HIV, antiplasmodial and antioxidant agents [14, 17].

Most microorganisms are developing resistance to the available antibiotics, therefore there is a need to discover and develop new antimicrobial drugs [6]. The ethanol root extract of *T. lupinifolia* reportedly showed good antimicrobial activity compared to

lupinifolin [14]. The leaf and stem extracts were not studied for biological activity, including antimicrobial and antioxidant activity. Therefore; study focuses on the extraction and fractionation of leaves and stems, determine their antimicrobial and oxidant activity and compare with the activity as reported on the roots of the same plant.

1.3 Objectives of the study

The objectives of the study were:

- a) To prepare and partially purify crude extracts from the stems and leaves of *T. lupinifolia*.
- b) To evaluate the antimicrobial and antioxidant activity of the crude extracts and fractions of the stems and leaves of *T. lupinifolia*.

1.4 Significance of the study

The validation of traditionally used medicinal plant for developing countries like Namibia is of great importance due to the reliance of indigenous communities on the use of herbal formulations. Phytochemical results from this study will support previous reports on the medicinal value of *T. lupinifolia* and contribute to its chemical knowledge of the chemical constituents of the plant as well as on drug discovery program.

1.5 Limitation of the study

A common problem encountered during phytochemical analysis is the formation of artifacts. These are compounds which are not biosynthesized by the plants but are formed when the natural product undergoes decomposition or a chemical reaction during the extraction and purification process. Crude extracts and fractions were kept in the fridge, that is, to avoid decomposition but the formation of artifacts during the

purification process could not be ruled out. A second limitation is the small quantities of metabolites that are normally isolated from plants, which in part could be due to the irreversible adsorption of metabolites on a solid support, for example, silica gel. This prevented the purification and full characterization of fractions. Although *T. lupinifolia* is used traditionally for the treatment of malaria like-symptoms, the fractions were not tested for antimalarial activity. This study only focused on the antimicrobial and antioxidant activity of the crude extracts and the fractions from the stems and leaves of *T. lupinifolia*.

2. CHAPTER TWO: LITERATURE REVIEW

2.1. Natural products

Natural products can be broadly classified into three groups namely: terpenes, phenolic compounds and alkaloids [1]. These compounds are not essential for the growth, development or reproduction of the plant, but they play a major role in the defense of plants against microorganisms [5]. Some secondary metabolites function as plant pigments, whereas others function as UV filters and antimicrobial agents [19, 20, 21]. Others play the role of chemical messengers, physiological regulators or cell cycle inhibitors in plants [20, 22]. Numerous compounds were also reported from *Tephrosia purpurea*, such as tannins, flavonoids and triterpenoid glycoside to mention few, which are reported as to be biologically active against numerous diseases [5].

Phenolic compounds are naturally occurring aromatic compounds which are widely spread throughout the plant kingdom [2]. They contain two carbon frameworks, namely the hydroxycinnamic and hydroxybenzoic structures [23]. Examples of phenolic compounds are benzoic acid derivatives, simple phenylpropanoids, anthocyanins, tannins, lignins and flavonoids [23, 24]. Flavonoids are the largest class of plant polyphenolic compounds which consist of two aromatic rings (A and B) and a heterocyclic ring (C) in their structures. They are classified based on their structures as flavones, flavonols, flavanones, flavanonols, flavanols, anthocyanidins, isoflavones and neoflavonoids as shown in figure 4 [19-21, 25, 26].

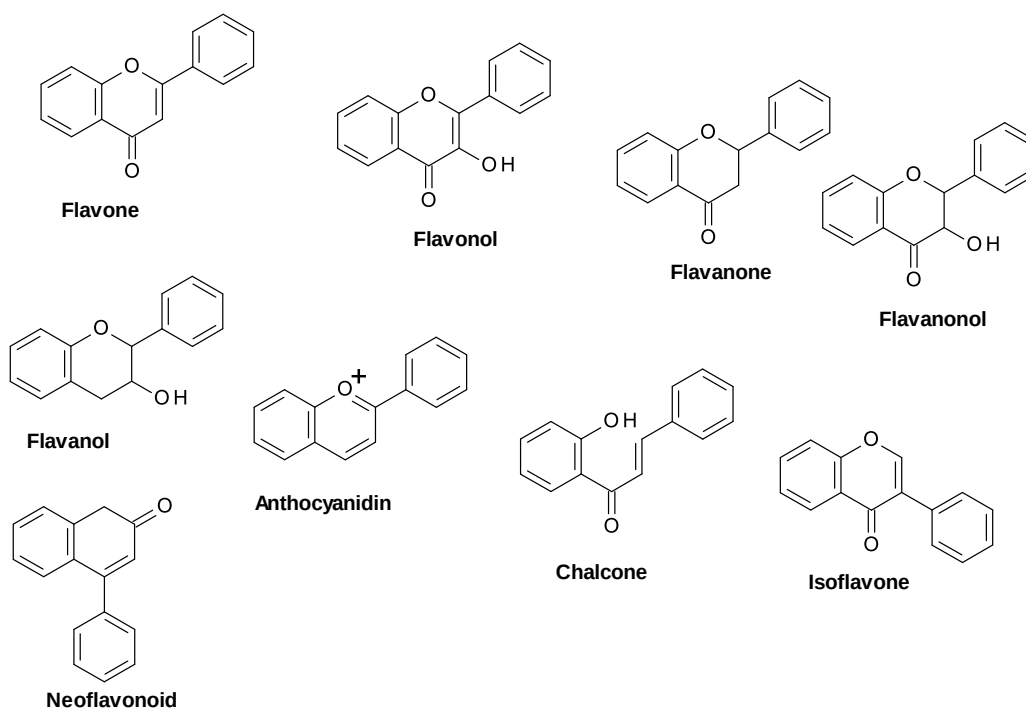


Figure 4: Classification of flavonoids [24].

2.2 Medicinal plants as a source of antimicrobial agents and antioxidants

Antimicrobial phytochemicals can be divided into several categories such as phenolics, essential oils, alkaloids and lectins as shown in figure 5 [5]. A phytochemical study on the butanolic seed extract of *T. purpurea* which is a species from genus *Tephrosia* led to the isolation of novel oleanene type triterpenoid glycoside and a saponin, which were reported to display antimicrobial activity against *S. pneumoniae* and *Alternaria alternata* [21, 27, 28]. The DCM extract of the roots and leaves, as well as the ethanol and aqueous seed extracts of *T. vogelii*, exhibited best antimicrobial activity against *S. aureus* and *E. coli* in comparison with other microbial strains [29]. Another study reported that the fruit, leaf and root extracts of *T. villosa* showed good growth inhibitory activity against *C. neoformans*, *E. coli* and *B. anthracis*, respectively [30].

Antioxidants are compounds that work by inhibiting oxidation of neutral molecules in the human body by interacting with free radicals to terminate the chain reaction [4, 7]. Another phytochemical analysis led to the isolation of a flavonoid, obovatin, which displayed antioxidant activity with an IC_{50} value of 3.370 $\mu\text{g/mL}$, from the roots of *T. toxicaria* [27]. In a separate study, lupenone was isolated from the methanol leaf extract of *T. villosa*, and identified as the compound responsible for the antioxidant activity and lipid peroxidation inhibitory activity displayed by the extract [30, 31].

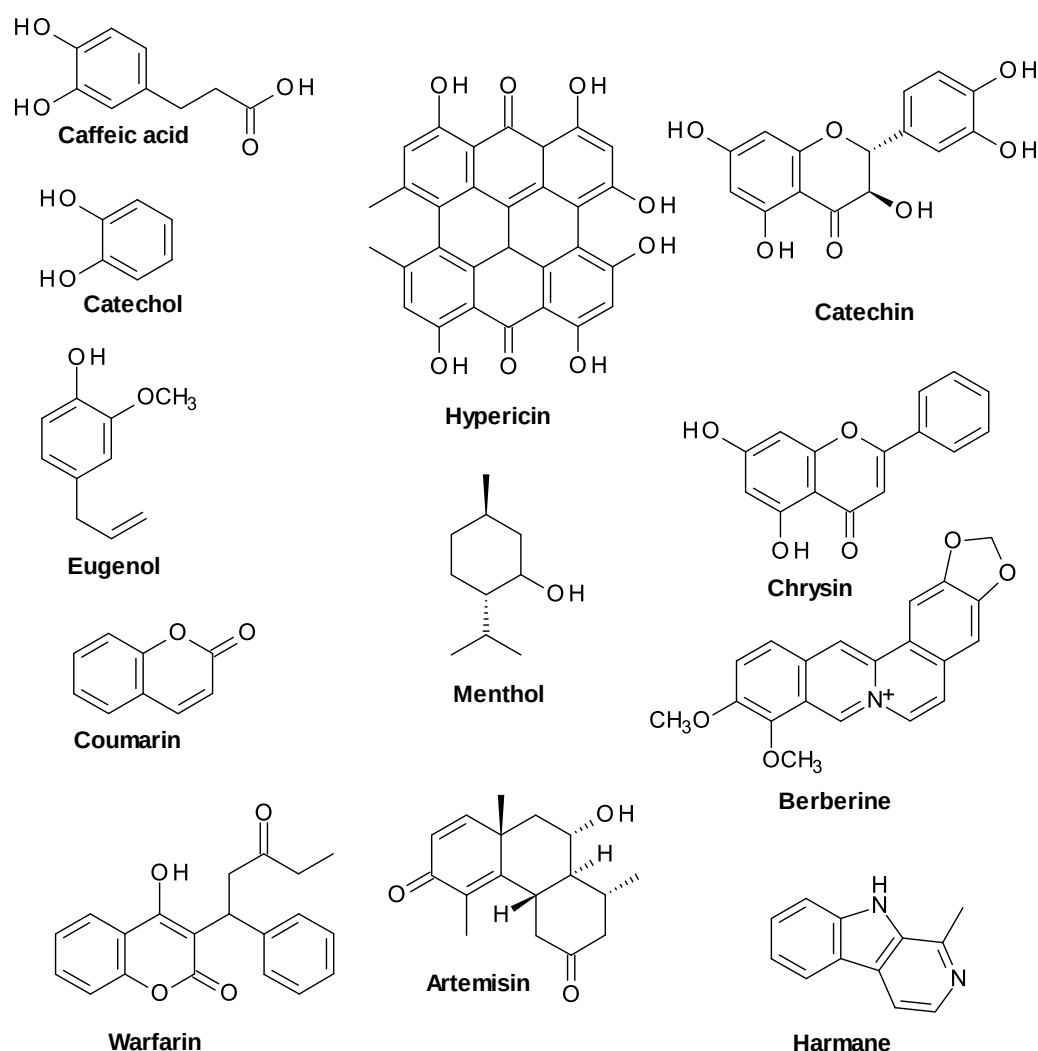


Figure 5: Structures of common plant chemicals that display antimicrobial activity [30].

2.3 Genus *Tephrosia*

Tephrosia is a member of the *Fabaceae* or *Leguminosae* family and it belongs to the *Mimosoideae* sub-family. It consists of about 630 genera and over 18,860 species of which 40 species are indigenous and 5 endemic to Namibia [9]. Species endemic to Namibia are *T. villosa*, *T. lupinifolia*, *T. dregeana*, *T. monophylla* and *T. pallida* [9]. It is the most economically important flowering plant family, distributed worldwide and is the third-largest in terms of number of species. *Tephrosia* species that are medicinally important and that have been extensively studied include *T. purpurea* distributed in India, Ceylon, Mauritius, Tropical Africa and subtropical regions, *T. pondoensis* found in South Africa, *T. odorata* and *T. socotrana* found in Yemen and *T. densiflora* which is endemic to west Africa [9].

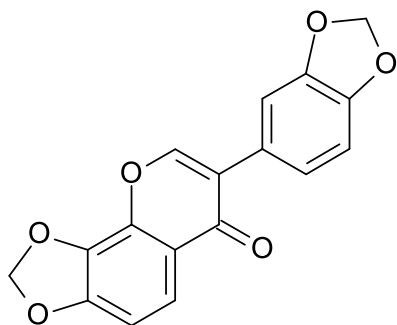
A report on the phytochemical analysis of *T. elata* seedpods revealed the presence of prenylated compounds such as rotenones, chalcones, and flavonoids [32, 33, 34]. In a separate study, qualitative screening tests were done on the methanol, ethanol, chloroform and hexane extracts of the leaves, stems and roots of *T. hookeriana* [36]. This study reported the presence of steroids, triterpenoids, alkaloids, flavonoids, saponins and tannins in all the plant parts [35, 36, 37]. Phytochemical study on the leaves and seeds of *T. purpurea* led to the isolation of tephrosin, rotenoid and other prenylated flavonoid derivatives [21, 38].

A quantitative analysis was carried out to determine the total phenolic and flavonoid contents of the hexane, ethanol, methanol and aqueous root extracts of *T. apollinea* [39- 41]. The total flavonoid contents of the hexane, water, methanol and ethanol extracts were reported as 66.95 ± 0.01 mg of QE/g extract, 23.58 ± 0.02 mg of QE/g extract, 10.71 ± 0.01 mg of QE/g extract and 78.09 ± 0.00 mg of QE/g extract, respectively. It was interesting to note that the ethanol extract showed the highest

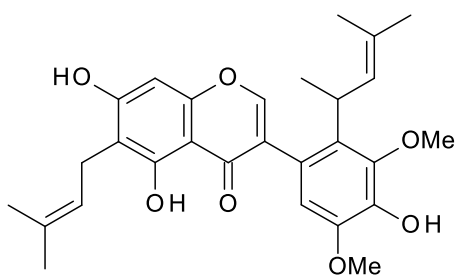
TFC of 78.09 ± 0.00 mg of QE/g extract, followed by the hexane extract of 66.95 ± 0.01 mg of QE/g extract, whereas the methanol extract had the lowest TFC of 10.71 ± 0.01 mg of QE/g extract [21, 42- 44].

Antimicrobial and antioxidant activity tests were done on other members of the genus *Tephrosia* [45]. Ethanol, aqueous and DCM root extracts of *T. vogelii* were prepared and screened for antimicrobial activity against *S. aureus*, *E. coli* and *Fusarium phoseolida*. All root extracts showed moderate inhibitory activity against the selected microorganisms [29, 45, 46]. The free radical scavenging activity of the methanol, ethanol and aqueous root extracts of *T. purpurea* was estimated using the DPPH free radical scavenging assay, with the aqueous extract showing the best antioxidant activity [27]. A separate study reported on the antibacterial activity of the methanol, ethanol and hexane root extracts of *T. hookeriana* against *Staphylococcus aureus*, *Aeromonas veronii*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Salmonella typhii*, using the agar disc diffusion method [38, 47, 48]. The methanolic root extract showed strong activity against *P. aeruginosa* at a concentration of 20 mg/ml with a zone of inhibition of 8-14 mm with [47- 49].

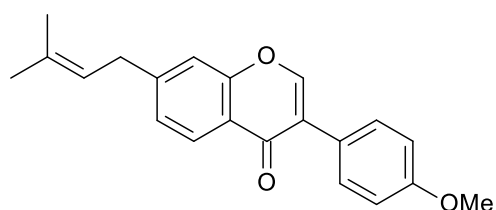
Other activities ascribed to members in this genus include antimalarial activity as evidenced by the isolation of a prenylated antiplasmodial flavone such as maxima isoflavone, pumilaisoflavone and 7,4-dihydroxy-3,5-dimethoxyisoflavone from the stem of *T. purpurea* as shown in figure 6[28].



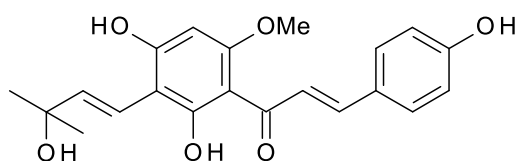
Maxima isoflavone



7,4-dihydroxy-3,5-dimethoxyisoflavone



Pumilaisoflavone



Candidachalcone

Figure 6: Flavonoids and chalcones isolated from the genus *Tephrosia* [27].

2.4 Biological assays

2.4.1 Antimicrobial assay

There are two common antimicrobial assays normally used in Biological laboratories, such as the agar-based diffusion and fluorescence-based assay.

The agar-based diffusion assay, also known as the disc diffusion method, is the method commonly used in clinical microbiology laboratories for antimicrobial,

antibacterial and antiplasmodial testing [6]. It is one of the simplest, more reliable and low-cost methods for determining the activity of samples against microbial strains such as *Bacillus cereus*, *Listeria monocytogenes* and *Escherichia coli* [50]. Despite these advantages, it is not an appropriate method to determine the minimum inhibitory concentration (MIC), because it is impossible to quantify the amount of antimicrobial agent diffused into the agar medium [50].

The fluorescence-based assay works by labeling bacteria with a cell-membrane-permeable, live-cell-staining or green fluorescent dye [50]. It is one of the most rapidly escalating microscopic techniques that allow the observation of microbes or bacterial vitality in live samples. It is more advantageous than the agar disc-diffusion method because the sample fixing is not required and it has a low probability of artifact formation. Shortcomings of this method include lower resolution power, dependency on the different dyes used, and it may produce a bias in the vitality quantification [50].

2.4.2 Antioxidant assay

The commonly used antioxidant assays in biological laboratories, are such as DPPH, Ferric reducing antioxidant power (FRAP), Oxygen radical absorbance capacity (ORAC), Malondialdehyde (MDA) and Cupric assay to mention few [51]. A study conducted on the determination of antioxidant using DPPH and reducing power tests revealed that the activity shown is due to the presence of flavonoids and phenolic compounds [52, 53].

There are two extraction techniques (using an orbital shaker or refluxing), normally used in the preparation of medicinal plant extracts in the determination of antioxidant activity [54- 57]. Extraction using an orbital shaker showed the highest TPC content

compared to the extraction using reflux method. This was attributed to the thermal decomposition that some of the phenolic compounds undergo when exposed to high temperatures. It is however reported that refluxing is a good extraction method for determination of antioxidants [53].

2.4.2.1 DPPH (2, 2-diphenyl-1-picrylhydrazyl radical) scavenging effect on the extracts

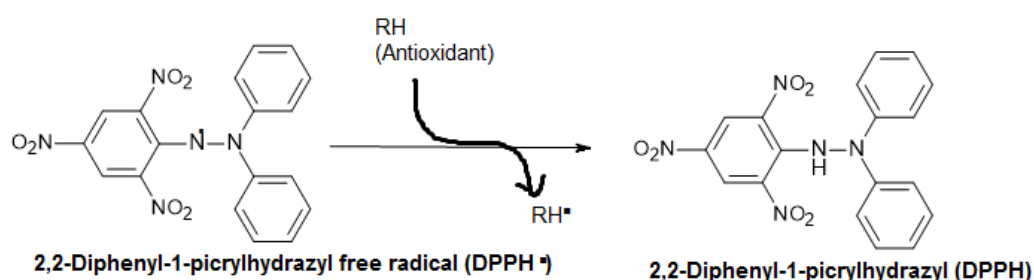


Figure 7: DPPH free radical conversion to DPPH by antioxidant compounds [37].

DPPH is a stable free radical which shows maximum ultraviolet and visible (UV-Vis) absorbance around 517 nm. Good scavenging activities are shown by a higher percentage of inhibition. Antioxidant activity of a sample is measured in terms of free radical scavenging activity. In the DPPH test, a test sample is subjected to DPPH free radical which will react with any antioxidant present in the sample. The free radical converts DPPH free radical into a neutral compound (DPPH). The degree of discoloration indicates the potential of free radical scavenging activity of the sample through hydrogen donation [37, 58].

2.4.2.1 Ferric reducing ability power assay (FRAP)

Ferric reducing the ability of plasma (FRAP) assay is one of the antioxidant assays used for the determination of antioxidant agents. This is antioxidant capacity assay which uses Trolox as a standard and is regarded as a novel method for assessing

"antioxidant power, which is based on the formation of O-Phenanthroline-Fe(2+) complex and its disruption in the presence of chelating agents [76].

3. CHAPTER THREE: RESEARCH METHODS

3.1 Collection and pre-treatment of plant material

The plant materials were collected from Tsumkwe in the Otjozondjupa region at the //Xaloba village (GPS = 19°37'30' S: 20°37'30' E). Taxonomic identification of the plant was done by Mr. David Aiyambo at the National Botanical Research Institute (NBRI). A voucher specimen was prepared and submitted. The leaves and stems were separated, washed with distilled water, air-dried for 24 hours, pulverized and stored at room temperature.

3.2 Phytochemical screening

3.2.1 Qualitative analysis of the root, leaf and stem extracts of *T. lupinifolia*

Qualitative screen tests were performed on the leaf, root and stem extracts of *T. lupinifolia* to detect the presence of the following phytochemicals: saponins, bitter principle, alkaloids, flavonoids, coumarins and terpenes. The roots leaf and stem extracts were tested using the methods as reported by Suman, *et al* [59].

3.2.1.1 Test for saponins

The leaf, root and stem extracts were prepared by macerating 10 g of the powdered plant material in 50 mL of ethanol and the extract was filtered, followed by concentration of the mother liquor under reduced pressure at 40 °C. The resulting extracts were diluted with 15 mL of distilled water in a test tube and heated on a water bath for 5 min. About 10 mL of the filtrate was transferred to a clean test tube and shaken for 10 sec. Formation of the honeycomb froth of height above 1cm for about 30 seconds indicated the presence of saponins [59].

3.2.1.2 Test for alkaloids

The root, leaf and stem extracts were prepared by mixing 1 g of the plant material in 5 mL of methanol, followed by 1 mL of a 10 % ammonia solution. The resulting mixture was refluxed for 10 min. A TLC of the extract was developed in ethyl acetate, methanol and water (10, 13.5:1). The developed plate was treated with Dragendorff's reagent, which was freshly prepared by dissolving 0.85 g of bismuth nitrate in 10 mL glacial acetic acid and 40 mL water under reflux. Formation of orange-brown spots indicated the presence of alkaloids [59, 60].

3.2.1.3 Test for flavonoids

The roots leaf and stem extracts were prepared by dissolving 1 g of the sample in 15 mL mixture of water and methanol (10 mL and 5 mL). The prepared extract was subjected to Shinoda's test, whereby small magnesium turnings were added to 3 mL of the filtrate followed by the drop-wise addition of concentrated HCl. The development of colors indicated the presence of flavonoids: orange color for flavones, red for flavonols and pink for flavanones.

3.2.1.4 Test for coumarins

One gram (1 g) of the plant material was extracted with 10 mL of DCM by reflux for 15 min. The filtrate was evaporated to dryness and the residue dissolved in 0.5 mL of toluene. A TLC was prepared and developed in toluene: ethyl acetate (93:7) solvent system and sprayed with 10 % ethanolic KOH. The formation of brown and yellow spots indicated the presence of coumarins.

3.2.1.5 Test for terpenes

The extract was prepared by mixing 1 g of the powdered plant material with 3 mL of chloroform. The mixture was stirred for 5 min followed by filtration and the filtrate

was treated with 10 drops of acetic anhydride and 2 drops of concentrated sulphuric acid. A reddish brown color shows the presence of terpenes.

3.2.2 Quantitative analysis of the methanol root, leaf and stem extracts of *T. lupinifolia*

3.2.2.1 Preparation of crude extracts and standard solutions

Crude extracts were prepared by soaking 50 mg of plant material (roots, leaves and stems) separately in 100 mL of methanol. The mixture was sonicated for 4 hours, filtered and stored in a refrigerator at 2-8 °C for further use. A standard gallic acid solution (25 mg/ml) was prepared by dissolving 25 mg of gallic acid in 1 mL of methanol and was used to determine the total phenolic content. The quercetin standard solution (10 mg/ml) was prepared by dissolving 100 mg of quercetin in 10 ml of methanol and was used for the determination of the total flavonoid content [21, 39, 41].

3.2.2.2 Determination of total phenolic content (TPC)

The total phenolic content of the root, leaf and stem extracts was determined by using the Folin Ciocalteu method [14, 26]. Samples (2 mL) were transferred into a clean test tube, followed by 1.0 mL of Folin- Ciocalteu's reagent and 0.8 mL of sodium carbonate (7%). The mixture was entirely mixed and allowed to stand for 30 minutes. The absorbance was recorded after 90 min at 760 nm using a UV spectrophotometer. A standard gallic acid curve was constructed by plotting concentrations of the prepared dilutions against their corresponding absorbance. Solutions of the extracts were prepared by dissolving 100 mg of the extract in 10 mL of methanol and treated in the same way as the standard solutions. The absorbance of each extract was obtained at 760 nm and their concentrations were determined using

the standard gallic curve. Total phenolic content was calculated and expressed as milligram gallic acid equivalents per gram dry weight (mg of GAE/g of DW) [52, 62].

3.2.2.3 Determination of total flavonoid content (TFC)

The total flavonoid content of the root, leaf and stem extracts was determined using the aluminum chloride complex forming assay [11, 62]. Four different concentrations of standard quercetin (2, 4, 6 and 8 mg/mL) were prepared in methanol. From each of the quercetin dilution, 100 μ L was pipetted separately and mixed with 500 μ L of distilled water, followed by 100 μ L of 5 % sodium nitrate. The resulting mixture was allowed to mix well and left standing for 6 minutes. Afterward, 150 μ L of 10 % aluminum chloride solution was added to each dilution and again allowed to stand for 5 minutes after which 200 μ L of 1 M sodium hydroxide was added. The absorbance was recorded after 90 min at 510 nm using a UV spectrophotometer. A standard quercetin curve was constructed by plotting concentrations of the prepared stock solution against their corresponding absorbance. The extract solutions were also treated in the same way as the standard solutions and absorbance were recorded at 510 nm [43, 52].

3.3 Materials and methods

All the materials and chemicals used in this study were purchased from Sigma-Aldrich, Merck Chemical (Pty) Ltd laboratory supplier division (Durban) and capital laboratory supplier CC (New Germany), namely: Silica gel (70-4230 mesh, Sigma-Aldrich), TLC aluminium sheets 20x20 cm silica gel 60 F₂₅₄.

The fractions were analysed on a gas chromatography-mass spectrometer (GC-MS, Agilent 7890 B GC system and Agilent 5977A (MSD)) analysis. GC-MS analysis of the fractions was done at the University of Namibia using a GC-MS 6800 equipped

with a fused silica capillary column DB-5 (3 m × 0.25 mm × 0.25 m). ¹H-NMR analysis was done at the Stellenbosch University in South Africa. The ¹H-NMR data were recorded using a Varian Spectrometer with operating frequencies of 400 and 600 MHz. Solvents used were deuterated CHCl₃ and DMSO. Chemical shifts (δ) were measured in parts per million (ppm) relative to the international standard tetramethylsilane (TMS). IR analysis of samples was done at the Namibia University of Science and Technology (NUST) using a Perkin Elmer spectrum two Fourier-transform infrared spectrophotometer. The melting points of the isolated compounds were determined using a Stuart melting point apparatus.

3.4 Separation methods

3.4.1 Analytical and preparative TLC

Alumina backed silica gel 60 F₂₅₄ coated TLC plates (Merck) was used to analyze the samples using different solvent systems as indicated in Table1 [55, 56]. Visualization of the UV active compounds was done by viewing the plates under an ultraviolet lamp at 254 nm and 366 nm wavelength while visualization of the UV inactive compounds was done by spraying the plates with *p*-anisaldehyde sulphuric acid, which was prepared by mixing 0.5 mL of *p*-anisaldehyde, 50 mL of acetic acid and 1 mL of concentrated sulphuric acid. After application of the spray reagent, the plates were heated at 100°C for about 5 minutes [44, 56]. The retention factors (R_f) were calculated using the formula:

$$R_f = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by solvent}}$$

Table 1: Solvent systems used for TLC

Solvent	Ratio
Ethyl acetate: hexane	1:1
Ethyl acetate: methanol	1:1
Ethyl acetate: methanol: water	4:0.5:0.5
Ethyl acetate: methanol: water	4:1:1
DCM: EA	3: 1
DCM: methanol: water	5:1:1
DCM	100%
Methanol	100%

Preparative TLC plates were prepared using high-purity grade (Merck grade 7749) silica gel. The slurry was prepared by dissolving 30 g of silica gel in 60 mL distilled water. TLC was prepared by coating 90 mL of the prepared slurry on a clean and dry glass plate (20 x 20 cm) and kept in a cupboard to dry. Separation of compounds was also done using commercial preparative TLC plates (PLC Silica gel 60 F₂₅₄, 1 mm thick, Merck). Activation of the commercial and preparative PLC plates was done by pre-heating the plates in an oven at 110°C for an hour and allowing it to cool to room temperature. Approximately 90 mg of the extract was loaded on the preparative TLC plate and developed using EA: MeOH: H₂O (4:0.5:0.5) solvent system [56].

3.4.2 Column chromatography

For column chromatography (CC), columns were packed with silica gel (70-230 mesh particles, Sigma-Aldrich) using the slurry packing method. Formation of bubbles in the prepared slurry was avoided by continuously stirring the slurry while pouring it into the column. The pre-adsorbed sample was introduced into the column and the column eluted with Ethyl acetate: Hexane mixture from (10: 90 to 90: 10)% [56].

3.4.3 Gas Chromatography-Mass Spectrometry (GC-MS)

The samples were prepared by dissolving 1 mg of the fraction in 1 mL of dichloromethane. One milliliter (1 mL) of the prepared solution was injected into the GC and vaporized in a heated chamber. Helium was used as the carrier gas which was set to beat 1 mL min⁻¹ and separation was done at a flow rate of 25 cm/sec. The injector temperature was adjusted at 250 °C, while the detector temperature was fixed to 280 °C. The separated compounds were then entered the mass spectrometer immediately that provides the mass spectrum of each individual compound [53, 54, 57].

3.4.4 Trituration

The leaf extract was subjected to trituration using the suspension method. The extract was suspended and stirred in 200 mL of EA followed by filtration. This was repeated three times. The extract was then suspended in 200 mL of methanol (3 times). The solutions obtained were combined based on their TLC profiles and concentrated using a rotary evaporator (Buchi) at 40°C [58].

3.5 Preparation of the crude stem and leaf extracts

3.5.1 Preparation of the stem extract

The powdered plant material (25.03 g) was macerated in 200 mL of dichloromethane: methanol mixture (1:1) on an orbital shaker for 24 hours at room temperature. The mixture was filtered and the residue was further extracted with 100 mL of MeOH for 12 hours. TLC profiles of the DCM: MeOH and MeOH extracts were obtained using ethyl acetate: hexane (1:3) as solvent system. The extracts were combined and concentrated using a rotary evaporator at 45 °C.

3.5.2 Preparation of the leaf extract

The powdered leaves (30 g) were macerated in 100 mL mixture of DCM: MeOH (1:1) using an orbital shaker for 24 hours at room temperature. The DCM: MeOH extract was first spotted on an analytical TLC plate to detect the number of compounds present. The dried DCM: MeOH extract was sequentially triturated three times with 200 mL of ethyl acetate and three times with 200 mL of methanol. The EA and MeOH extracts were analyzed using analytical TLC plate for further purification.

3.6 Fractionation of the crude leaf and stem extracts

The crude leaf and stem extracts were subjected to preparative TLC for separation of compounds as outlined in section 3.2.1.1. The crude leaf extract was purified using a mixture of EA: MeOH: H₂O (4:0.5:0.5) as a solvent system while the crude stem extract was eluted using DCM: EA (3:1).

3.7 Analysis of the fractions

The resulting fractions were analyzed using a combination of GC-MS and ¹H-NMR [54-58].

3.8 Biological testing

3.8.1 Antimicrobial activity testing

The fractions, as well as the crude leaf and stem extracts, were tested for antimicrobial activity using the agar disc diffusion method against four microbial strains; *Klebsiella pneumonia*, *Escherichia coli*, *Candida albicans* and *Staphylococcus aureus*. Ampicillin (1 mg/mL) was used as positive control. Agar plates were prepared by pouring agar solution into a sterile petri-dish. The plates

were allowed to solidify overnight in a bacteria free area [64-67]. The microbial strains (*K. pneumonia*, *E. coli*, *C. albicans* and *S. aureus*) were first activated in the broth and incubated at 37 °C for 24 hours. After 24 hours, 100 µL of the strains were separately inoculated on the agar plate. Solutions of the isolated metabolites as well as the leaf and stem extracts were prepared by dissolving 10 mg of each sample in 1 mL of dimethyl sulfoxide (DMSO). Twenty microlitres (20 µL) of the prepared solution was dropped directly on top of the paper disc and it was placed on the prepared agar plate. The plates were allowed to settle for an hour for the sample to diffuse into the paper and the plates were then incubated at 37 °C for 24 hours. After 24 hours of incubation, the zones of inhibition were measured and recorded in millimeter (mm) [68, 69].

The antimicrobial activities of the tested compounds were compared to the antibiotic standard ampicillin [70]. Antimicrobial activity of a compound was indicated by an average zone of inhibition. The zone of inhibition was measured using a transparent 300 mm ruler and the strength of activity was classified as follows: for diameter ≥ 17 mm (strong), a diameter ranging from 11–17 mm (moderate), a diameter ranging from 6-11mm (weak) and diameter ≤ 6 mm (negative) [44]. Each test was done in duplicate.

3.8.2 Determination of the minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of the isolated metabolites that showed antimicrobial activity against four selected microbial strains was determined. Six different concentrations of the fractions were prepared: 10, 8, 4, 2, 1 and 0.5 mg/mL. The MIC testing was done following the procedures reported by Collins *et al* [67].

3.8.3 Antioxidant activity testing

The free radical scavenging activities of the fractions were determined using the DPPH free radical scavenging assay as described by Mulla *et al* [43]. The DPPH solution (0.012 mg/mL) was prepared by dissolving 1.18 mg of DPPH in 100 mL of ethanol. The different concentrations (5.000, 2.500, 1.250, 0.625, 0.313, 0.156, 0.078, 0.039 mg/mL) of each fraction were prepared as follows: A volume of 100 μ L of the DPPH solution was added to each of the prepared concentrations. The mixtures were homogenized thoroughly and incubated for an hour at room temperature. The absorbance of the resulting solution was measured at 517 nm using a Beckman model DU-40 spectrophotometer and ascorbic acid was used as a standard. The percentage inhibition of the DPPH radical was calculated by comparing the results of the tested fraction with that shown by the control using the equation below [47, 71-74].

$$\text{Percentage inhibition} = \frac{1 - (\text{absorbance of test})}{\text{absorbance of control}} \times 100$$

Antioxidant data was analyzed as percentage inhibition using the DPPH method and expressed as 50 % inhibitory concentration (IC₅₀), which was computed using Microsoft Excel 2010.

4. CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Qualitative phytochemical analysis

The qualitative screening on leaves and stems of *T. lupinifolia* confirmed the presence flavonoids, alkaloids, terpenoids and coumarins in leaves while only alkaloids, terpenoids, saponins and coumarins were present in stems. The presence of saponins in stems was shown by the formation of the honeycomb froth of height above 1cm. Formation of orange-brown spots on the TLC, after treated with Dragendorff's reagent, confirmed the presence of alkaloids in the leaves. The presence of flavonoids in all the plant parts was indicated by the formation of orange, red and pink colors after the addition of HCl, which was identified as flavones, flavonols and flavanones as indicated by the colors observed. The results are in agreement with the previous studies done on the roots of *T. lupinifolia* by Smalberger [14], which reported on the isolation of two flavonoids.

Phytochemical screening reveals the presence of some biochemical compounds. Development of brown and yellow spots on TLC indicated the presence of coumarins while the presence of terpenes was presented by the reddish-brown colors. The screening confirmed the presence of flavonoids, coumarins and terpenes in the leaves, roots and stems. Phytochemical screening done on other species of the genus *Tephrosia*, revealed the presence of saponins, alkaloids and flavonoids [4]. Flavonoids, saponins and alkaloids are well known for their wide range of pharmacological activities such as anticancer, antimicrobial and antiplasmodial [4].

Table 2: Phytochemical screen test results for the leaf, root and stem extracts of *T. lupinifolia*.

Phytochemical constituents	Leaves	Roots	Stems
Flavonoids	+++	+	++
Alkaloids	+++	++	-
Terpenoids	+	++	+++
Saponins	-	+++	++
Coumarins	+++	++	+

+++ : Strong color intensity, ++: Medium color intensity, +: Weak color intensity, -: Color not detected

4.2 Quantitative phytochemical analysis

The root, leaf and stem extracts were investigated for phenolic and flavonoid contents. Samples were prepared according to the method outlined in section 3.8.2. The phenolic and flavonoid contents were determined using the standard curve shown in Figure 8 and 9, respectively.

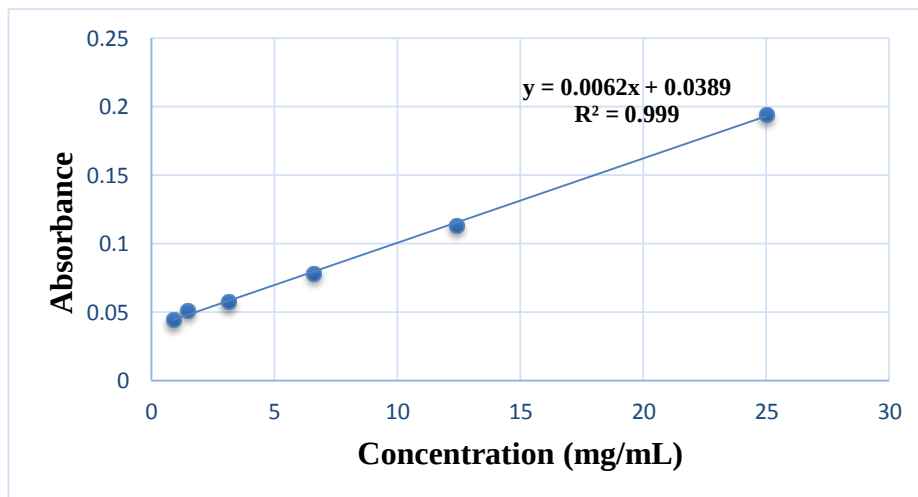


Figure 8: Standard curve for the Folin Ciocalteu assay using gallic acid as standard.

Figure 9 displays a plot of the standard quercetin curve, which provided the equation to be used for determining the TFC in the leaves and stems of *T. lupinifolia*.

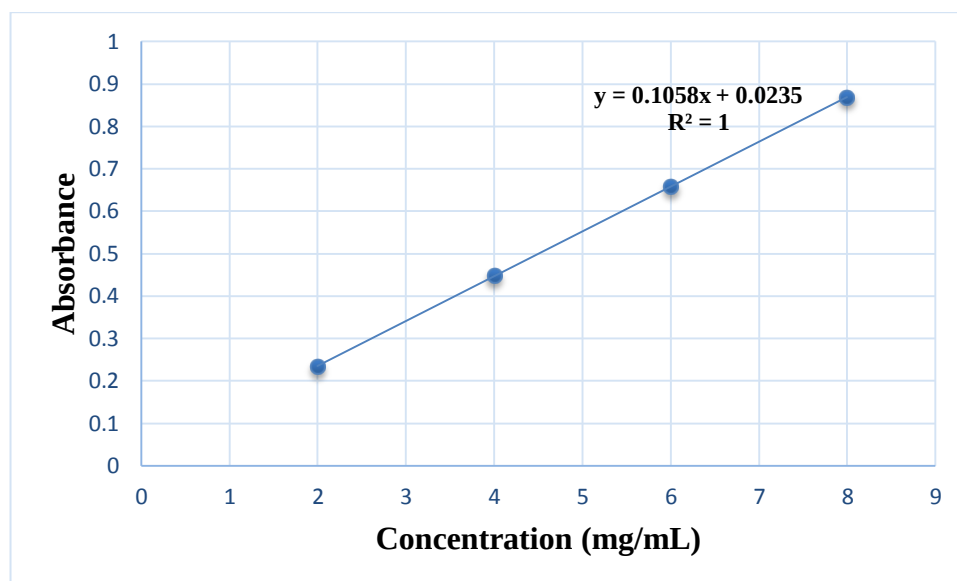


Figure 9: Standard curve for the aluminium chloride assay using quercetin as standard.

The total phenolic content (TPC) was expressed as gallic acid equivalents (Table 3). The TPC in the roots, leaves and stems of *T. lupinifolia* was observed in the range of 45.3 ± 0.001 mg of GAE/g to 250.5 ± 0.001 mg of GAE/g extract. The highest total phenolic content was recorded for the stem extract as 250.5 ± 0.001 mg of GAE/g extract and the lowest in the leaf extract as 45.3 ± 0.001 mg of GAE/g extract. However, the leaf extract gave the highest flavonoid content of 32.8 ± 0.032 mg of QE/g extract, whereas the lowest was found in the root extract as 13.2 ± 0.003 mg of QE/g extract.

According to the qualitative screen test (Table 2) the flavonoid content is higher in the leaves followed by the stems and the roots and this result correlated with the quantitative results. The quantitative results revealed the highest TFC of 32.8 ± 0.032 mg QE/g from leaves extract and the lowest in the root extract as shown in Table 3.

Table 3: Total phenolic and total flavonoid contents of the roots, leaves and stems of *T. lupinifolia*.

Plant part	TPC (mg of GAE/g extract)	TFC (mg of QE/g extract)
Leaves	45.3 $\pm$ 0.001	32.8 $\pm$ 0.032
Roots	58.3 $\pm$ 0.001	13.2 $\pm$ 0.003
Stems	250.5 $\pm$ 0.001	20.3 $\pm$ 0.013

The results are presented as mean $\pm$ SEM. Each experiment was repeated two times; (n =2).

4.3 Fractionation of the stem and leaf extracts

After extraction, the concentrated crude extract was subjected to preparative TLC in order to purify and separate compounds into fractions, which results in nine fractions. These fractions were tested for antimicrobial activity against various micro-organisms as well as for antioxidant activity. The fractions show good biological activity in comparison to the crude extract. Crude extract is a combination of compounds with several biological activities, fractions contain a minimum number of compounds, therefore, performed better biological activity than the crude extract.

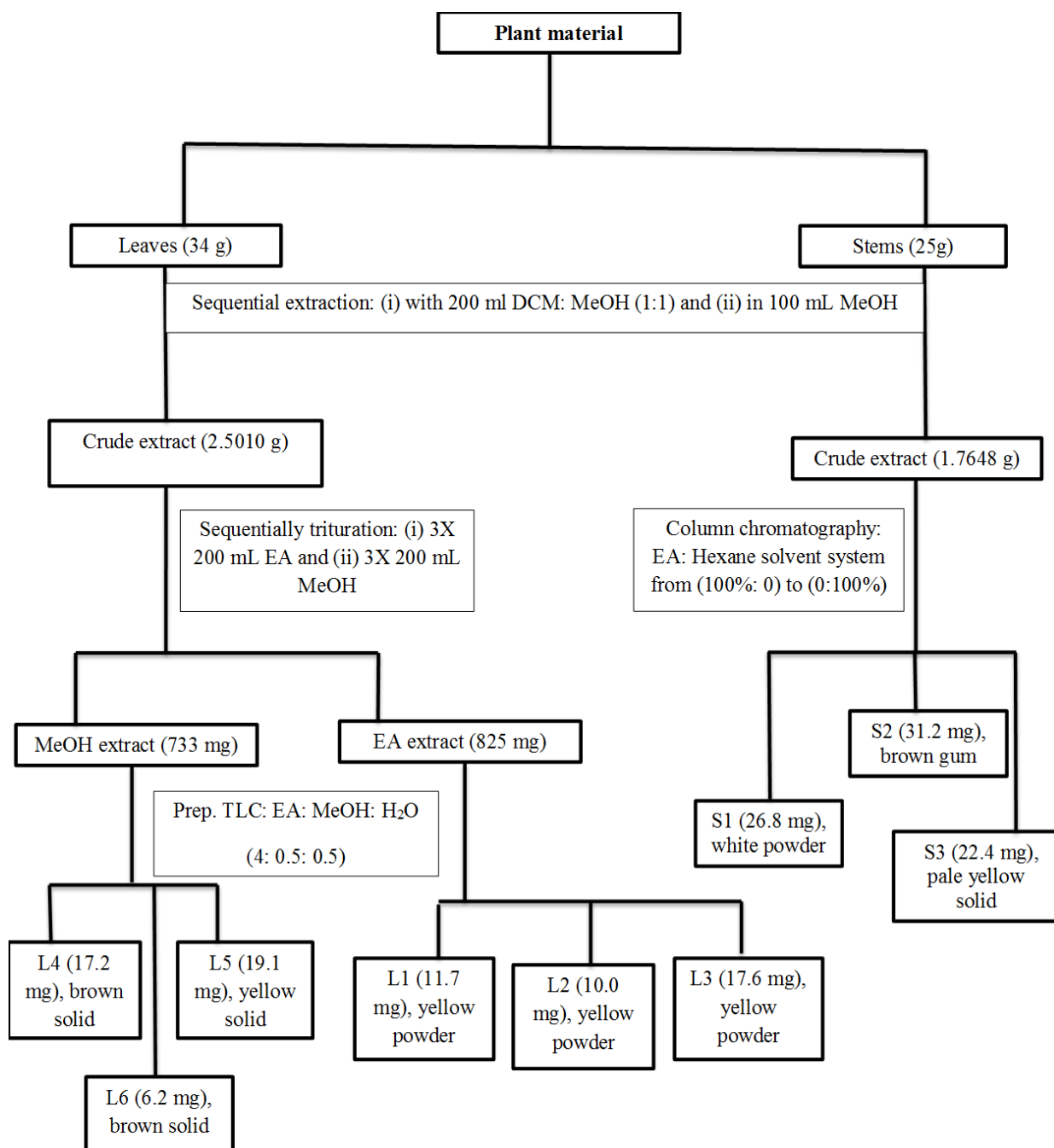


Figure 10: Flow chart showing the purification of the crude leaves and stems extracts of *T. lupinifolia* to yield fractions L1-L6 and S1-S3, respectively

The chromatographic purification of the crude leaves and stems extracts of *T. lupinifolia* led to the following fractions; S1-S3 were obtained from the crude stems extract and L1-L6 from the leaves extract.

Table 4: Yield of fractions obtained from the crude leaf and stem extracts of *T. lupinifolia*.

Plant part	Code	Yield (mg)
Stems	S1	26.8
	S2	31.2
	S3	22.4
Leaves	L1	11.7
	L2	10.1
	L3	17.6
	L4	17.2
	L5	19.1
	L6	6.2

Figure 11 shows the TLC profile of the fractions from the leaves and stems of *T. lupinifolia* developed in DCM: EA (3:1) solvent system. TLC profile of DCM/EA (3:1) showed that fractions S3, L1-L3 display more or less the same TLC profile, but their TLC profile does not correlate with the results obtained from the GC-MC analysis as shown in Figure 11 and appendix.

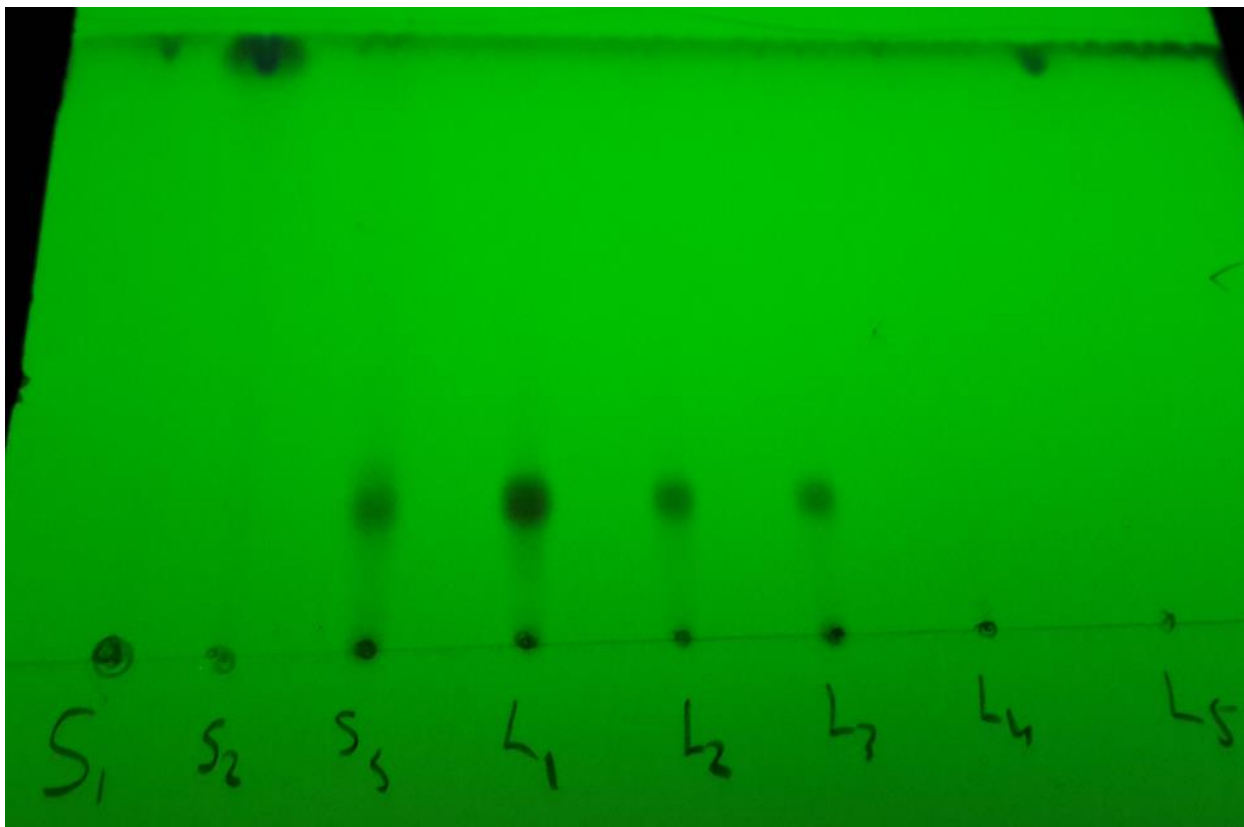


Figure 11: A TLC plate showing the fractions obtained from the crude leaf and stem extracts of *T. lupinifolia*, developed in DCM: EA (3:1).

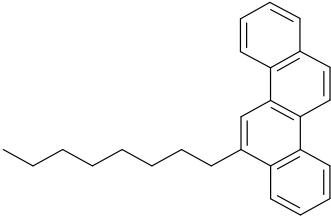
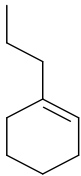
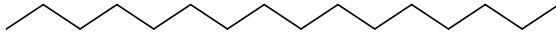
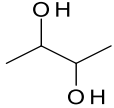
4.4 Analysis of the fractions

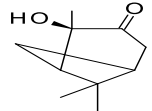
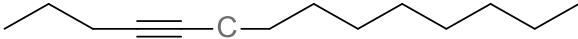
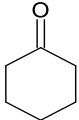
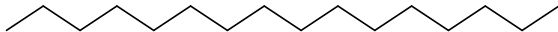
4.4.1 GC-MS analysis

The fractions were subjected to GC-MS analysis, which revealed that the fractions were partially purified, for instance: S2, L1 and L3 comprised of 2-3 compounds present in different proportions (Figures 17, 19, 21), L5 comprised of 10 compounds (Figure 23) while S3 and L6 consisted of 2 compounds as shown in Figure 18 and 24 in the appendix. Tentative identification of major compounds in the fractions was carried. GC-MS results revealed that the fraction L6 consists of two compounds: (i) hexadecane, an alkane hydrocarbon with molecular formula $C_{16}H_{34}$, was identified by analyzing fraction L6 on GC-MS together with the standard alkane mixture under the same conditions (retention time), (ii) cyclohexanone was also tentatively identified from L6 through a NIST library search for matching mass spectrum.

Apart from the two compounds identified from L6, fractions S2, S3, L1, L3, L4 and L5 were also analysed using GC-MS. Compounds were tentatively identified by matching the mass spectra obtained with that of known compounds stored in the National Institute of Standards and Technology (NIST) library. Results obtained are shown in Table 5. The GC chromatogram of fraction L2 revealed that it composed of a single compound which is relatively pure, but this does not clarify the purity, the fraction may contain nonvolatile substances which were not detected by GC-MS.

Table 5: Major compounds which were tentatively identified from the fractions of the leaves and stems of *T. lupinifolia*

Fraction code	Compound	Exact mass (g/mol)	Structure
S2	6-octylchrysene	340.500	
S3	1-propyl-1-cyclohexene	124.223	
L1	Hexadecane	226.441	
L3	2,3-butanediol	90.121	

L4	2,5,5-trimethylbicyclo[3.1.1]heptan-3-one	152.233	
L5	4-tetradecyne	194.362	
L6	Hexadecane and Cyclohexanone	226.441 and 98.143	 cyclohexanone  hexadecane

4.4.2 ¹H-NMR analysis

The fractions were also subjected to ¹H-NMR analysis. The NMR spectrums show that the fractions are partially purified and their spectrums were not interpretable due to the overlapping and unresolved signals as shown in Figure 15 and 16 in the appendix.

4.5 Biological activity

4.5.1 Antimicrobial activity

4.5.1.1 Agar disc-diffusion method

The fractions from the crude stems and leaves were subjected to antimicrobial activity testing against four selected microbial strains: *K. pneumonia*, *E. coli*, *C. albicans* and *S. aureus*, but L5 and L6 were not tested for antimicrobial activity due to very low yields obtained. The crude leaf, crude stem, S1 and L4 showed activity against all strains. However, S2 and S3 did not show activity against *K pneumonia*. Similarly, antimicrobial activity was not detected for S3, L1 and L3 against the fungal strain, *C. albicans*. The result shows that S3 was the least active against all strains. Overall, the crude stem extract showed better antimicrobial activity (12 mm inhibition zones) suggesting that the compounds in the crude extract work synergistically to enhance the antimicrobial effect. On the other hand, fraction L4 also showed similar inhibition zones (highest of 13 mm and lowest of 8 mm) with the crude stem extract (highest of 12 mm and lowest 7 mm) and could be targeted for further investigation.

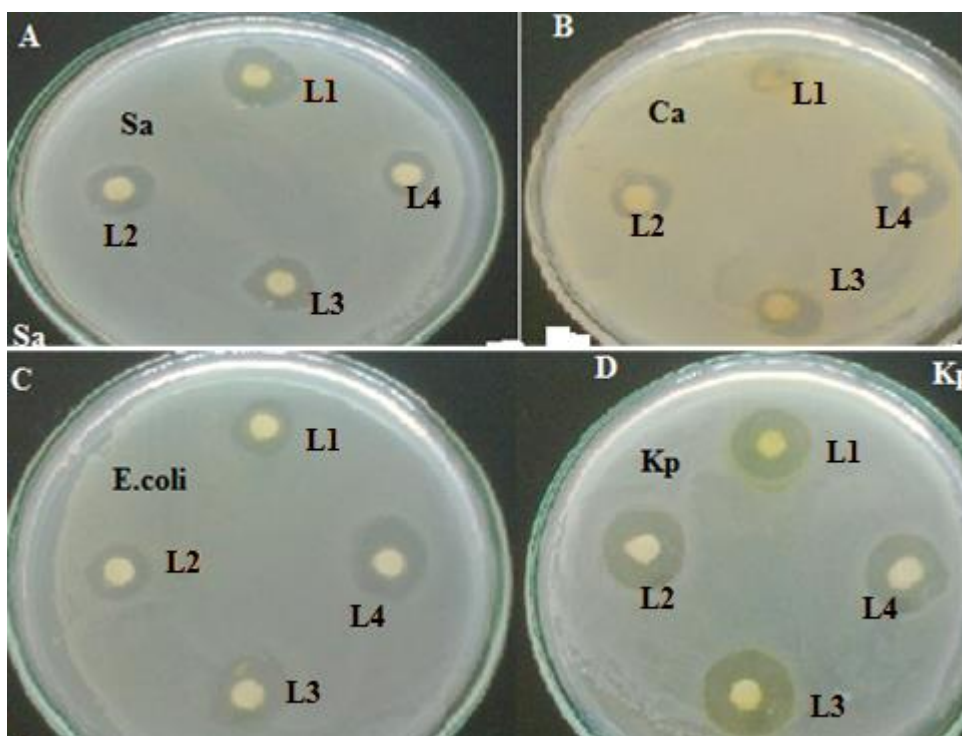


Figure 12: A culture plate showing the zones of inhibition of microbial growth for fractions L1 - L4 against *S. aureus* (A), *C. albicans* (B), *E. coli* (C) and *K. pneumoniae* (D).

Phenolic compounds are ubiquitous secondary metabolites in plants, they are known to possess antioxidant activity and antimicrobial activity, therefore the biological activities displayed by the leaf and stem extracts may be due to the presence of these compounds [61]. The study carried out by Smalberger [14], resulted in the isolation of lupinifolin, which was subjected to the antimicrobial activity test together with the ethanol root extract of *T. lupinifolia*. Lupinifolin showed the best inhibitory activity. The fractions and extracts tested also displayed good inhibitory activity (≥ 14 mm) against the four selected microbial strains [14]. Table 7 below summarizes the antimicrobial activity of the fractions against the selected microbial strains.

Table 6: Antimicrobial activity of the crude root and leaf extracts as well as the fractions of *T. lupinifolia*

Sample	Concentration (mg/mL)	Zone of inhibition (mm)			
		<i>S. aureus</i>	<i>E. coli</i>	<i>K. pneumonia</i>	<i>C. albicans</i>
Crude leaf	10	6	10	6	10
Crude stem	10	7	9	14	12
S1	10	8	6	8	10
S2	10	10	5	ND	7
S3	10	10	7	ND	ND
L1	10	9	6	6	ND
L2	10	ND	7	9	10
L3	10	8.5	11	11	ND
L4	10	8	10	10	13
Ampicillin	10	20	19	19	18

ND = Not Detected

4.5.1.2 Minimum inhibitory concentration (MIC)

The lowest concentrations of S1- S3 and L1- L4 that showed growth inhibitory activity against the selected microbial strains were determined. As reported by Collins *et al* [67], compounds that displayed strong inhibitory activity at a very low concentration are regarded to be good antimicrobial and antioxidant agents [67]. A MIC of 20 mg/mL was recorded for fraction S2, which showed that it is a poor microbial growth inhibitor compared to the other fractions.

Table 7: Minimum inhibitory concentration of the fractions against four microbial strains .

Compound	MIC (mg/mL)			
	<i>S. aureus</i>	<i>K. pneumonia</i>	<i>E. coli</i>	<i>C. albicans</i>
S1	10	4	10	10
S2	20	ND	20	20
S3	4	20	8	ND
L1	10	4	10	ND
L2	ND	2	ND	10
L3	2	2	2	ND
L4	2	2	2	2

4.5.2 Antioxidant activity

Antioxidant activity of the fractions (S1-S3, L1, L5 and L6) was determined using DPPH as a stable free radical, which shows maximum ultraviolet and visible (UV-Vis) absorbance around 517 nm. The results showed the *in vitro* antioxidant activity of the fractions in comparison to vitamin C. The highest scavenging activity of 89, 87 and 90% inhibition was displayed by S1, L5 and L6, respectively at the highest concentration of 5 mg/mL. At 5 mg/mL, L6 displayed a % inhibition (90%) greater than that of the positive control. As expected, the antioxidant activity of the isolated metabolites revealed that when the concentrations of samples are gradually increased, there is an increase in the % inhibition. The antioxidant data showed that *T. lupinifolia* is a source of antioxidant compounds since the aerial parts of the plant (stems and the leaves) displayed good antioxidant activity; therefore, they serve as potential sources of antioxidants.

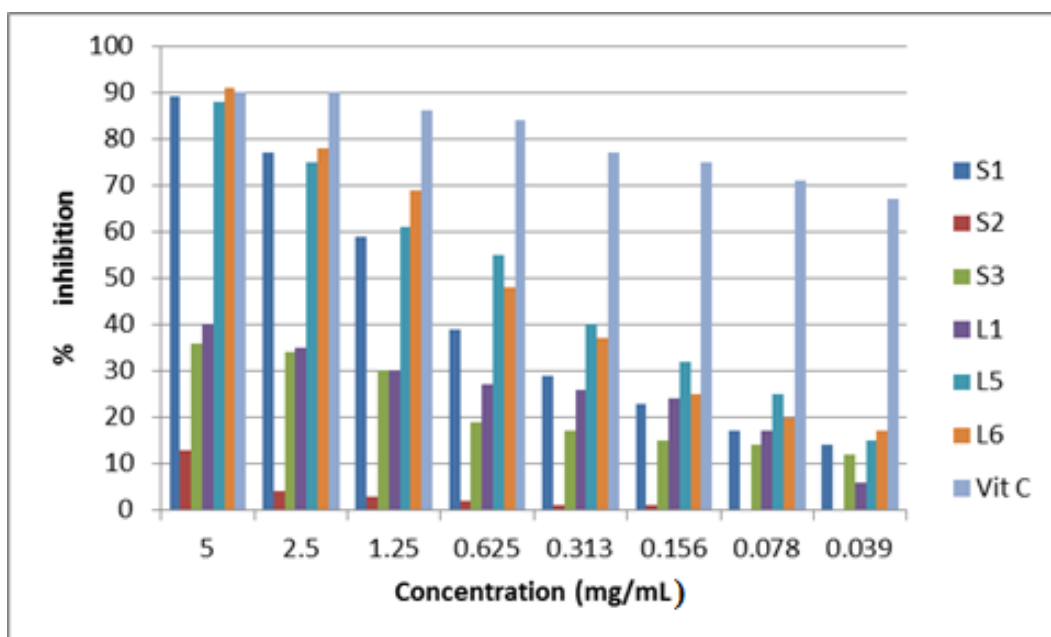


Figure 13: Percentage inhibition of the free DPPH radical displayed by fractions from *T. lupinifolia*.

The ability of samples to scavenge the DPPH radical was measured on the basis of their concentrations providing the 50 % inhibition (IC_{50}). The radical scavenging ability, IC_{50} values of the fractions and vitamin C are presented in figure 14 below. A smaller IC_{50} value is an indication of good antioxidant activity [75]. Fractions S1, L1, L5 and L6 showed the best antioxidant activity with IC_{50} values of 2.3060 ± 0.0920 , 4.1120 ± 0.0815 , 4.2210 ± 0.1085 and 0.7551 ± 0.0111 mg/mL respectively. Fraction L6 displayed the lowest IC_{50} value of 0.7551 ± 0.0111 mg/mL; therefore showed the best antioxidant activity compared to the other fractions and merits further purification.

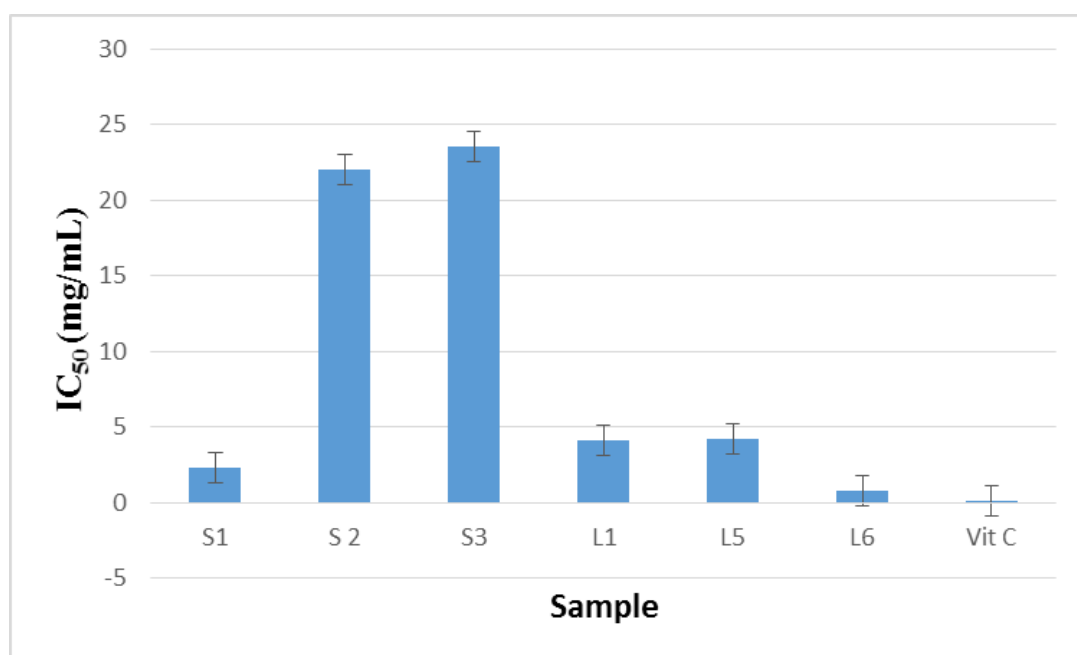


Figure 14: IC_{50} values indicating antioxidant activities of the fractions of *T. lupinifolia*.

5. CHAPTER FIVE: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The objectives were to prepare and partially purify (fractionate) the crude extracts from the stems and leaves of *T. lupinifolia*. These were then further evaluated for antimicrobial and antioxidant activities. As mentioned in section 1.5, the yields of the fractions which varied from 10.1 mg for L2 to 31.2 mg for S3, was too little to continue with the isolation and full characterization of metabolites. The study reported on the antioxidant and antimicrobial activity of the nine fractions (S1, S2, S3, L1, L2, L3, L4, L5 and L6) from the leaves and stems of *T. lupinifolia*. GC-MS analysis revealed that the fractions vary in terms of their compositions. Seven compounds were tentatively identified from leaf and stem fractions.

The phytochemical screen tests revealed the presence of flavonoids, alkaloids, saponins, terpenoids and coumarins in the plant. All of the afore-mentioned classes of phytochemicals were present in the roots, but flavonoids were absent in stems while saponins were not detected in the leaves. Phytochemical screening of other species of the genus *Tephrosia* revealed the presence of the same phytochemical classes as reported in this study.

Alkaloids and saponins are known for their ability to inhibit the growth of gram positive and gram negative bacteria, it is, therefore, possible that the observed antibacterial activity displayed by fractions and the crude extracts can be ascribed to the presence of alkaloids, flavonoids and saponins.

The fractions, as well as the leaf and stem extracts, were tested for antimicrobial and antioxidant activity. The crude stem extract showed the highest inhibition zone against *K. pneumonia*, followed by fraction L4 which showed inhibitory activity

against *C. albicans*. Some components of the fractions were identified using GC-MS. Fraction S1 and L6 showed low IC₅₀ values and therefore displayed the highest antioxidant activity.

The formations of artifacts are detected when the partially purified fractions spotted alongside the crude extracts on an analytical TLC plate. New spots were observed in the fractions which could not be detected in the crude extracts, but were separated using preparative TLC method. The study intended to isolated pure compounds and to subject them to one - and two- dimensional NMR. Only some analyses were carried out due to the small quantities obtained. For example, L1 and L6 were obtained in yields of 11. 7 mg and 6.2 mg, respectively.

5.2 Recommendations

It is recommended that isolation of pure metabolites from the leaves and stems of the *T. lupinifolia* should be done to allow full characterization of metabolites. The study revealed that the crude leaf and stem extracts, as well as the fractions, exhibited antimicrobial activity against some gram-positive and gram-negative bacteria. However, further purification of the fractions will improve their antimicrobial and antioxidant activity as shown in table 6 and figure 14 . Fraction S1, S2, S3, L3 and L3 are good antimicrobial agents while fraction S1, L1, L5 and L6 are good antioxidant agents. Further purification may result in the isolation of novel antimicrobial and antioxidant agents. Quantitative chemical tests confirmed the presence of phenolics and flavonoids in the leaves, roots and stems. Considering the therapeutic value of the flavonoids it is worth isolating and confirming the presence of these and/or other flavonoids in the stems and leaves of *T. lupinifolia*.

6. CHAPTER SIX: REFERENCES

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APPENDIX

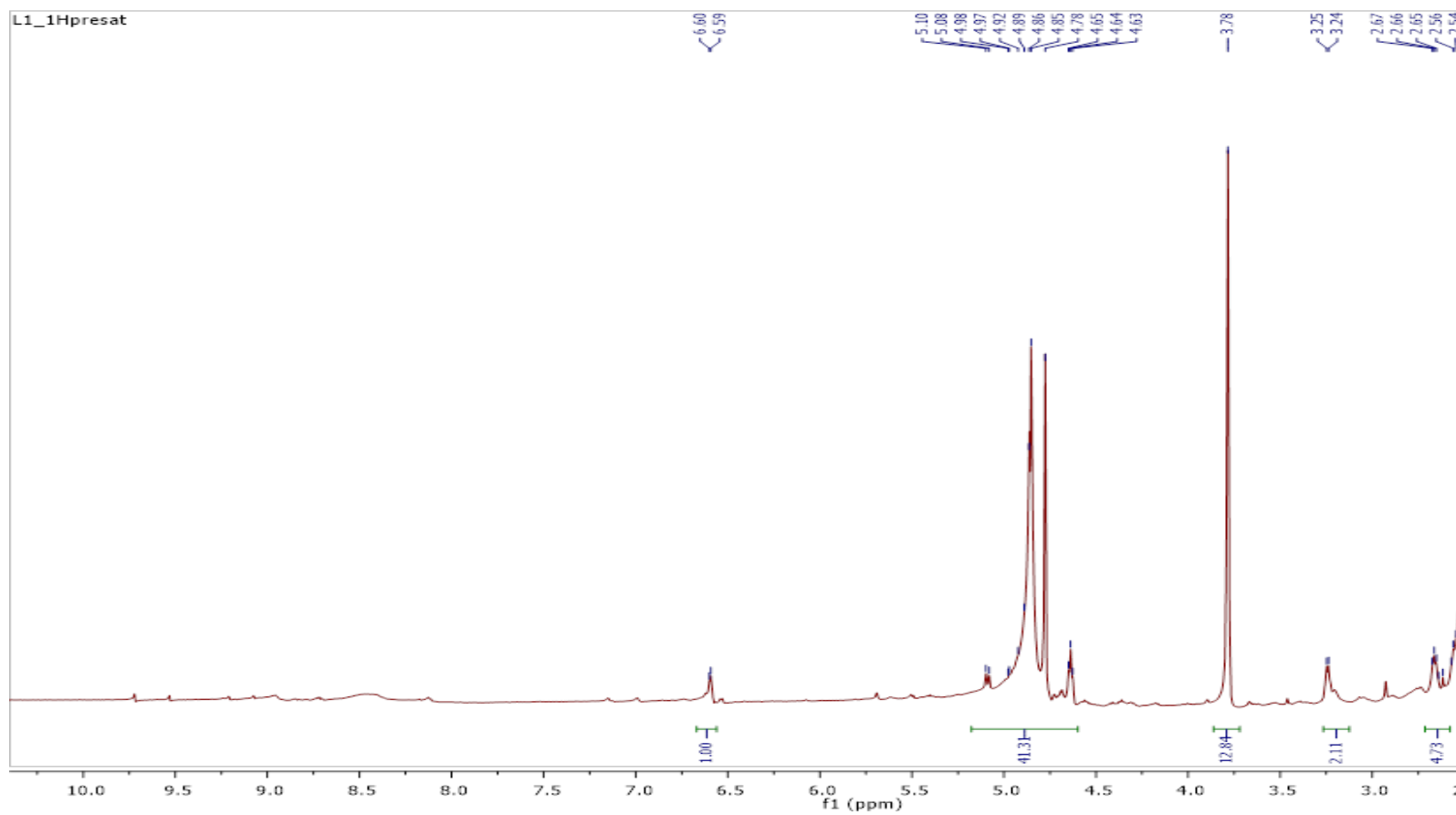


Figure 15: ^1H -NMR spectrum of fraction L1 in DMSO-d_6 at 600 MHz

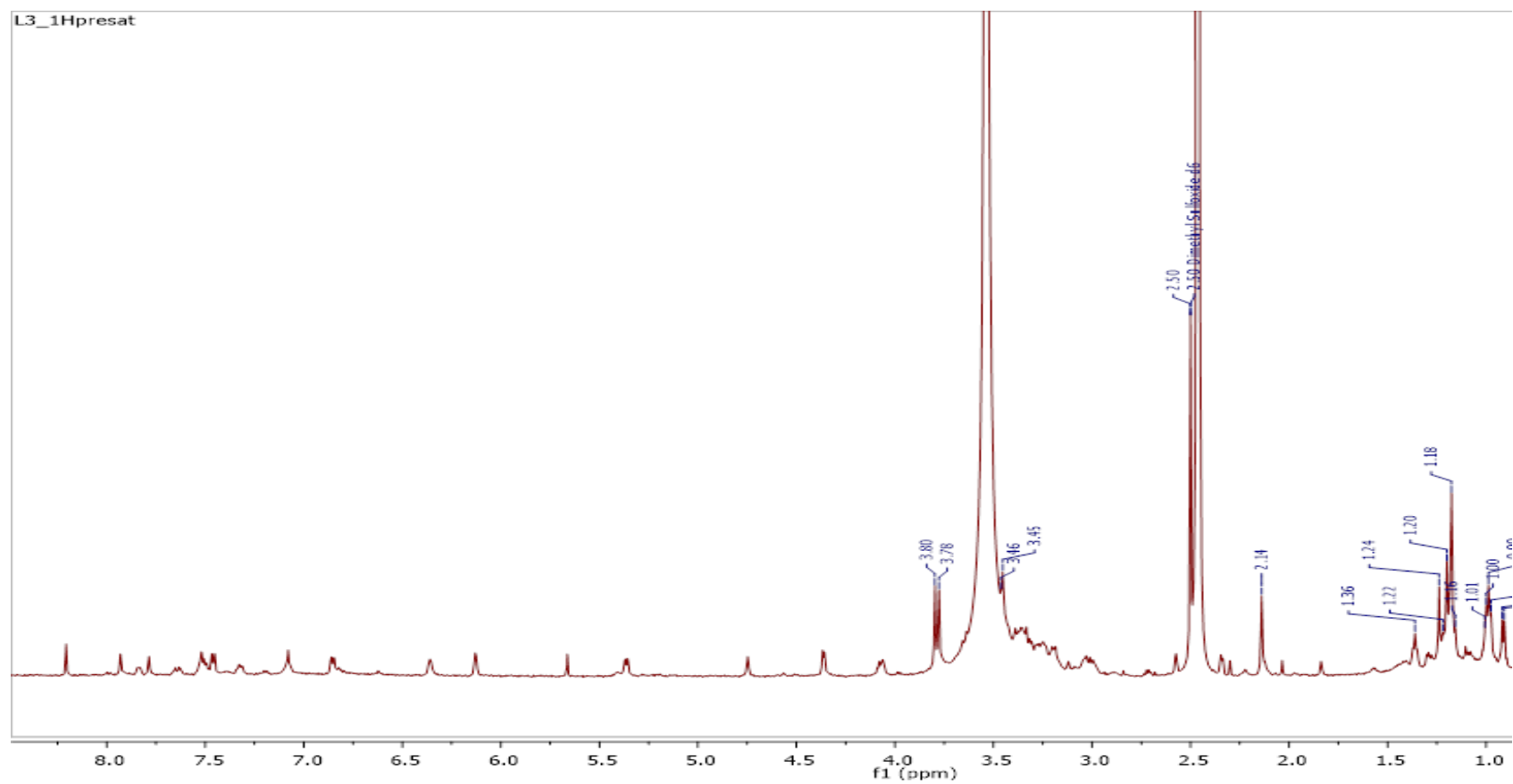


Figure 16: ^1H -NMR spectrum of the fraction L3 in DMSO-d_6 at 600 MHz

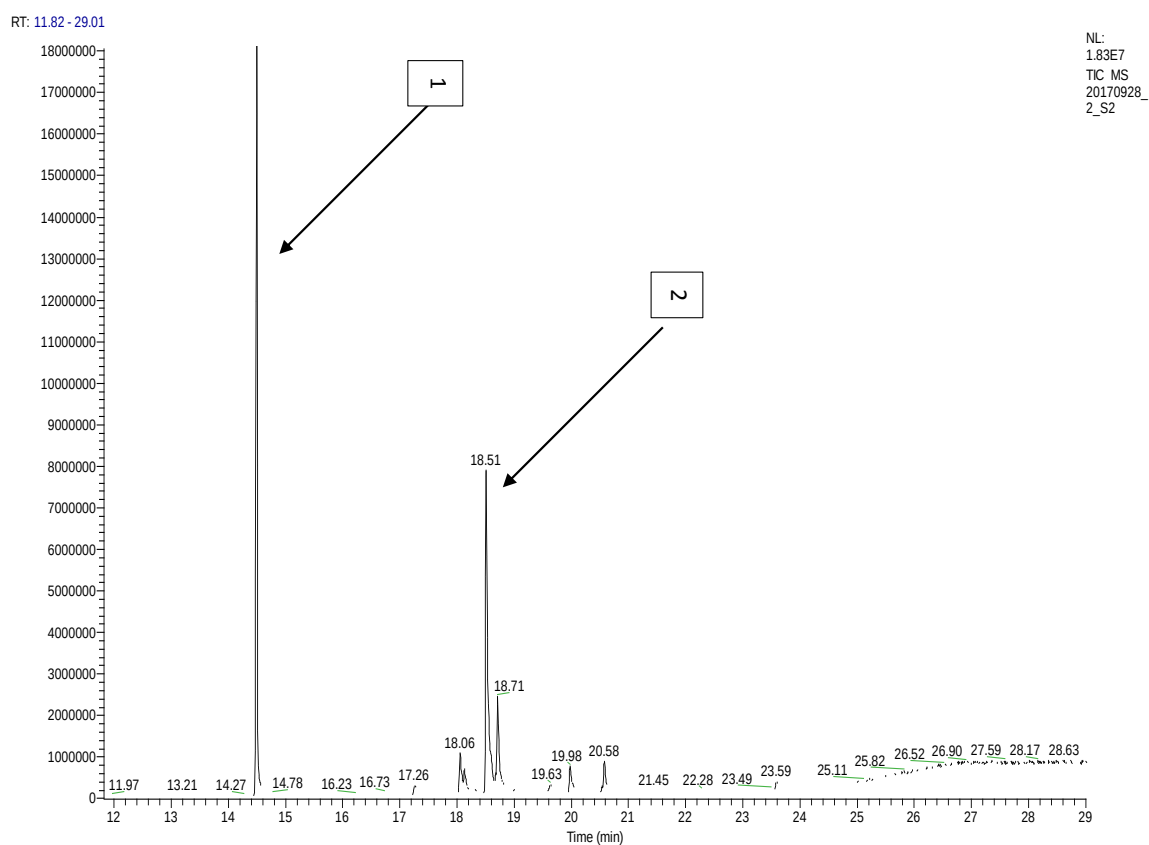


Figure 17: GC chromatogram of fraction S2 from the stem extract of *T. lupinifolia*

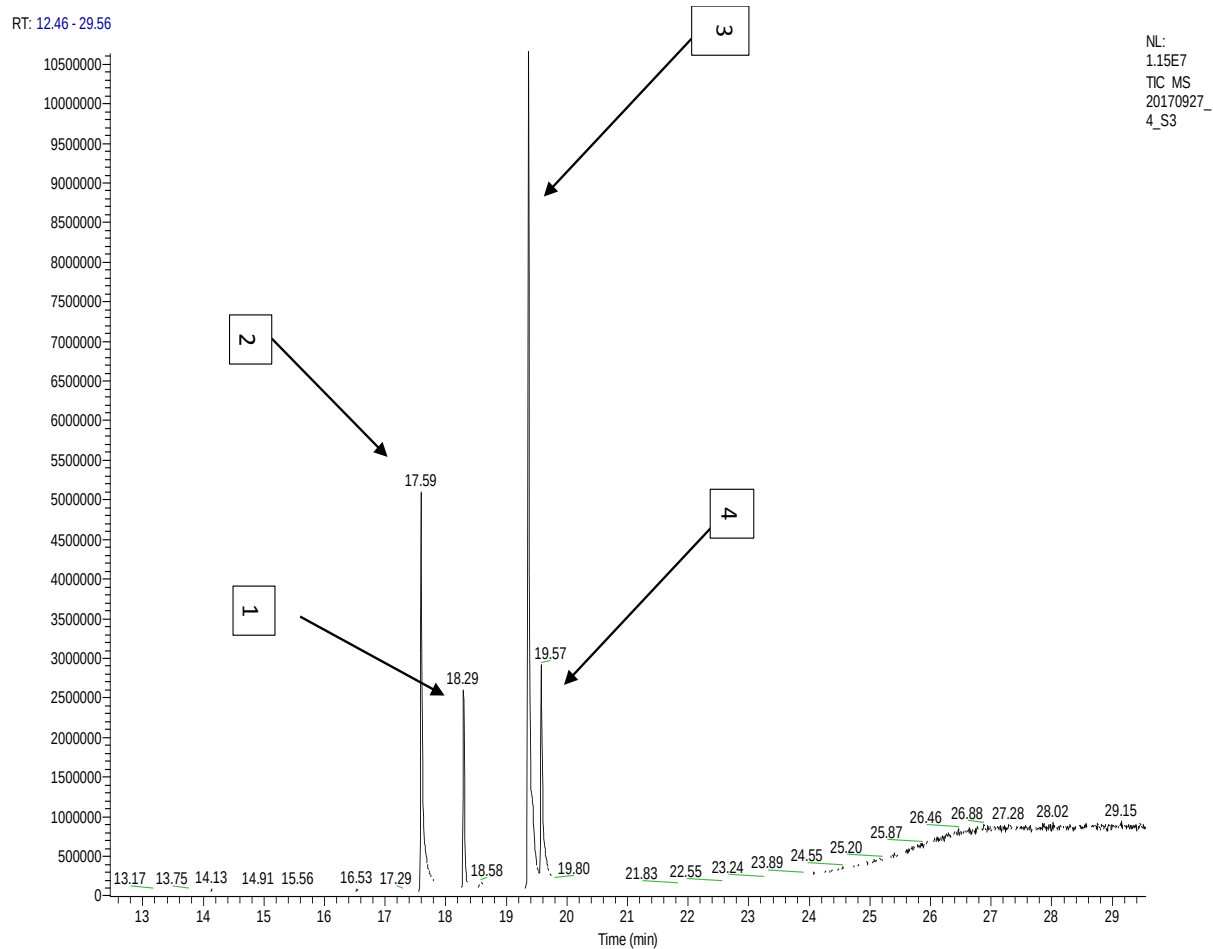


Figure 18: GC chromatogram of fraction S3 from the stem extract of *T. lupinifolia*.

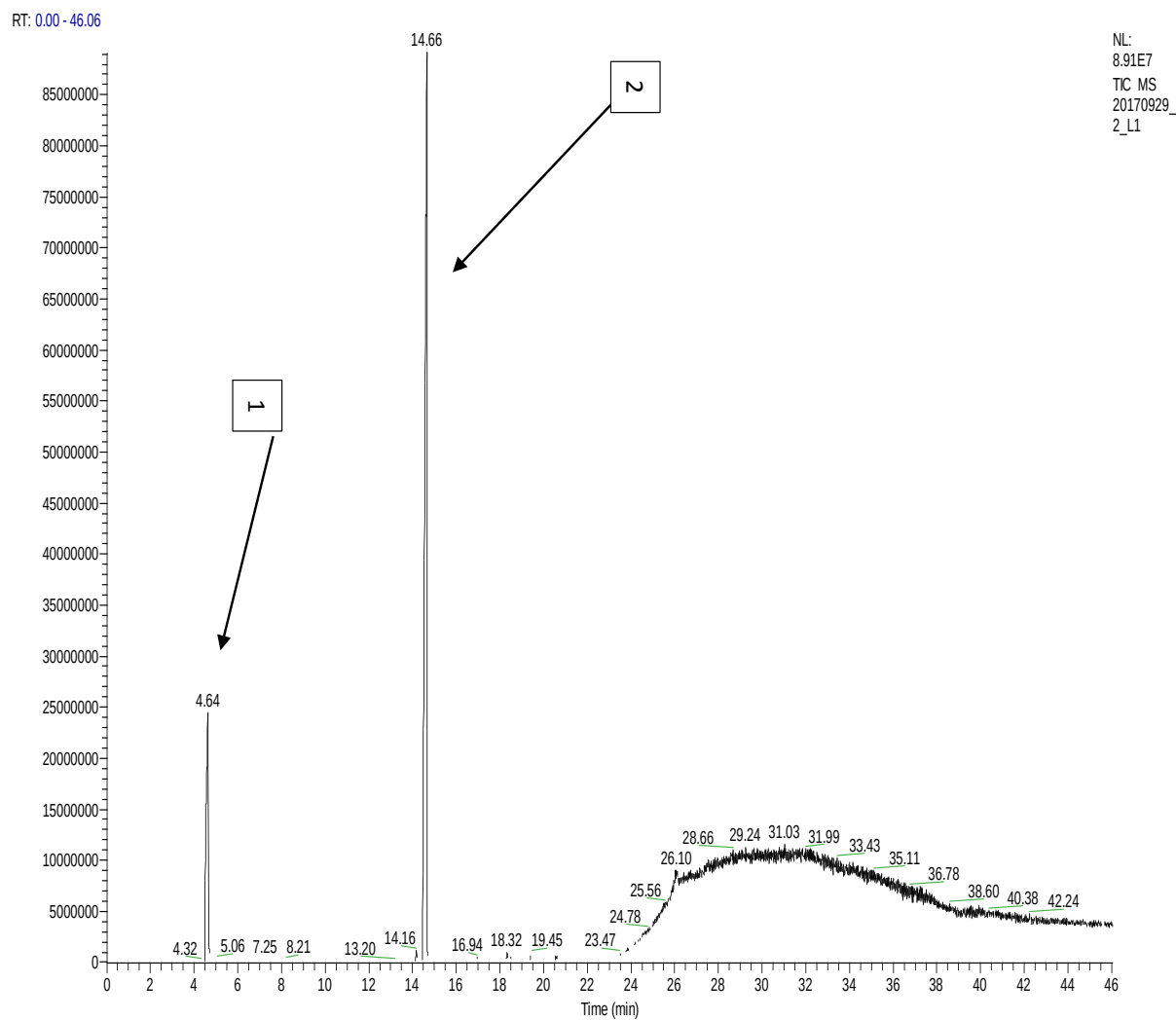


Figure 19: GC chromatogram of fraction L1 from the leaf extract of *T. lupinifolia*.

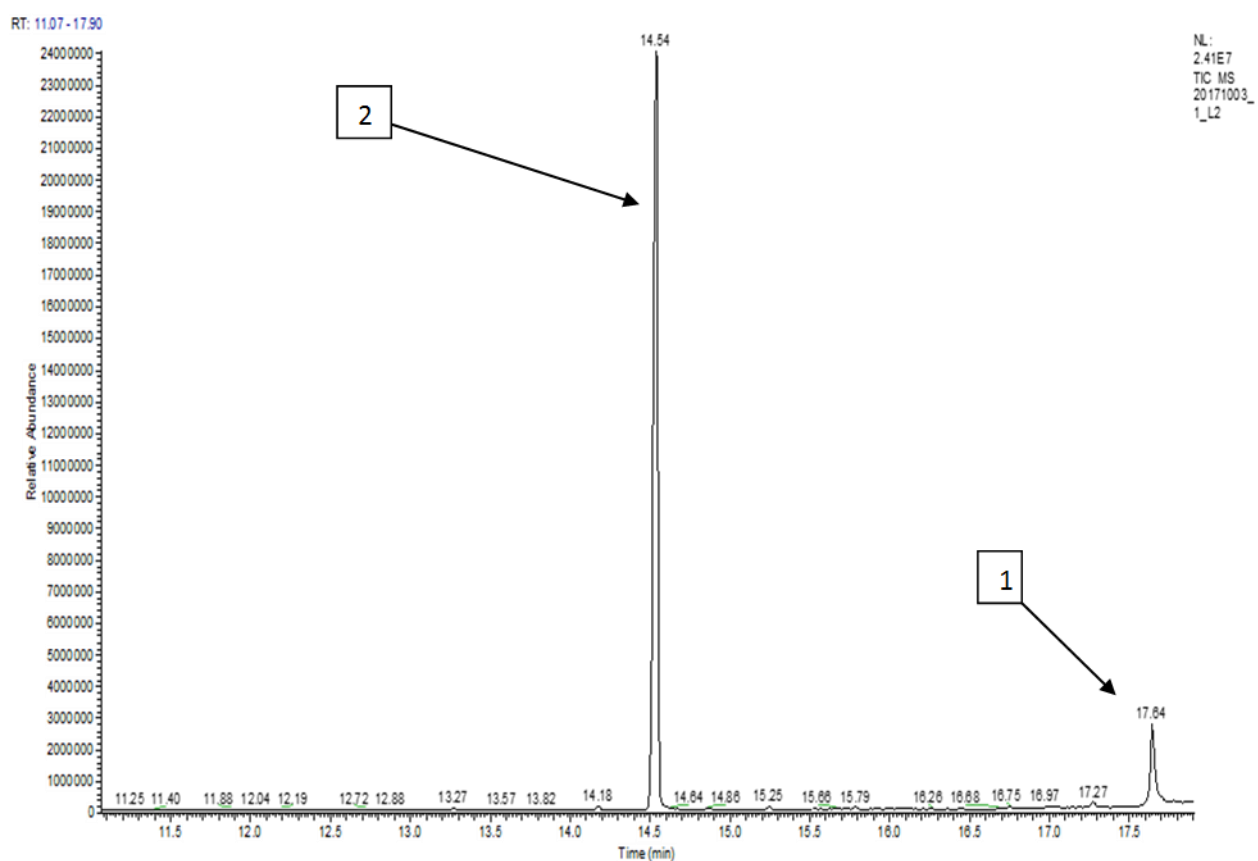


Figure 20: GC chromatogram of fraction L2 from the leaf extract of *T. lupinifolia*.

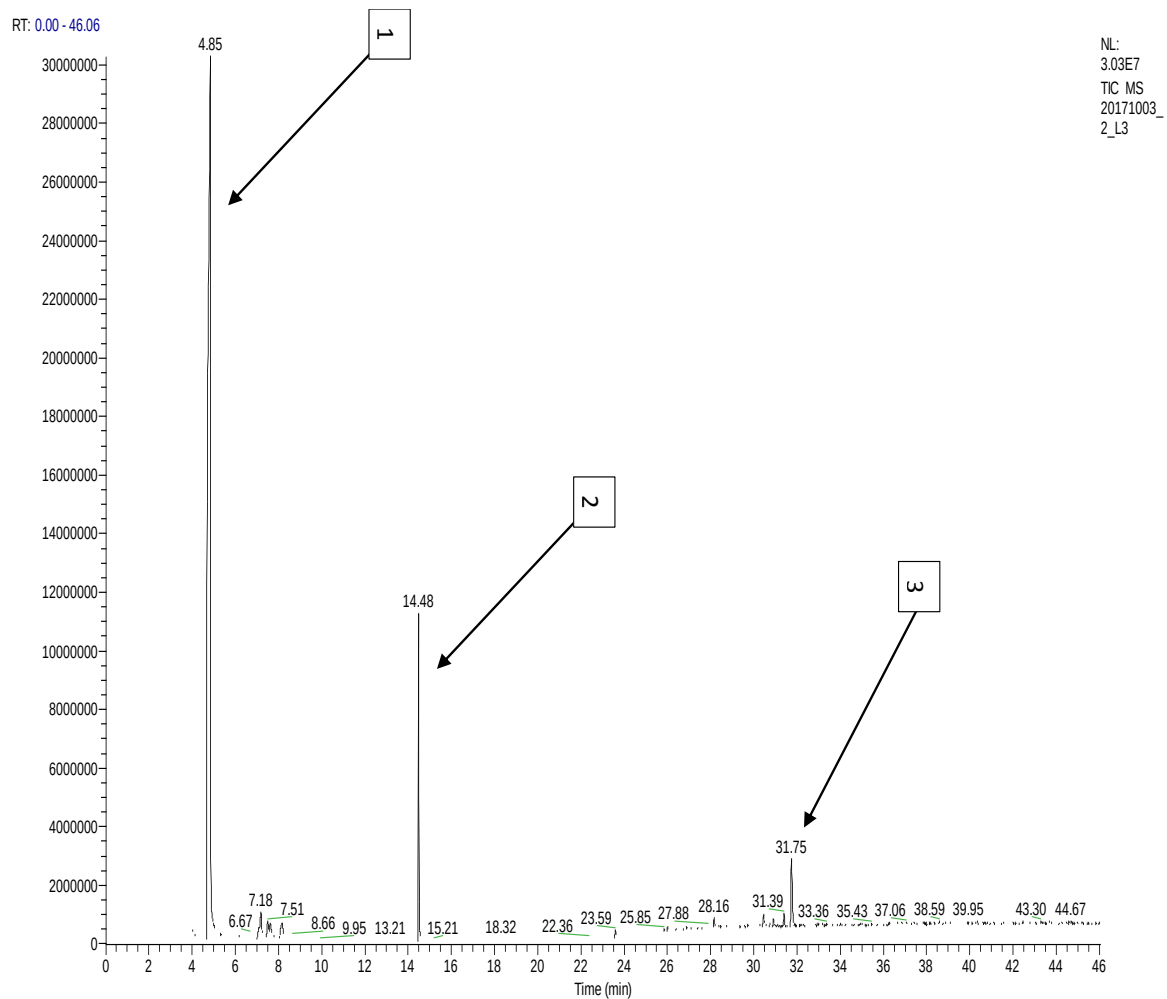


Figure 21: GC chromatogram of fraction L3 from the leaf extract of *T. lupinifolia*.

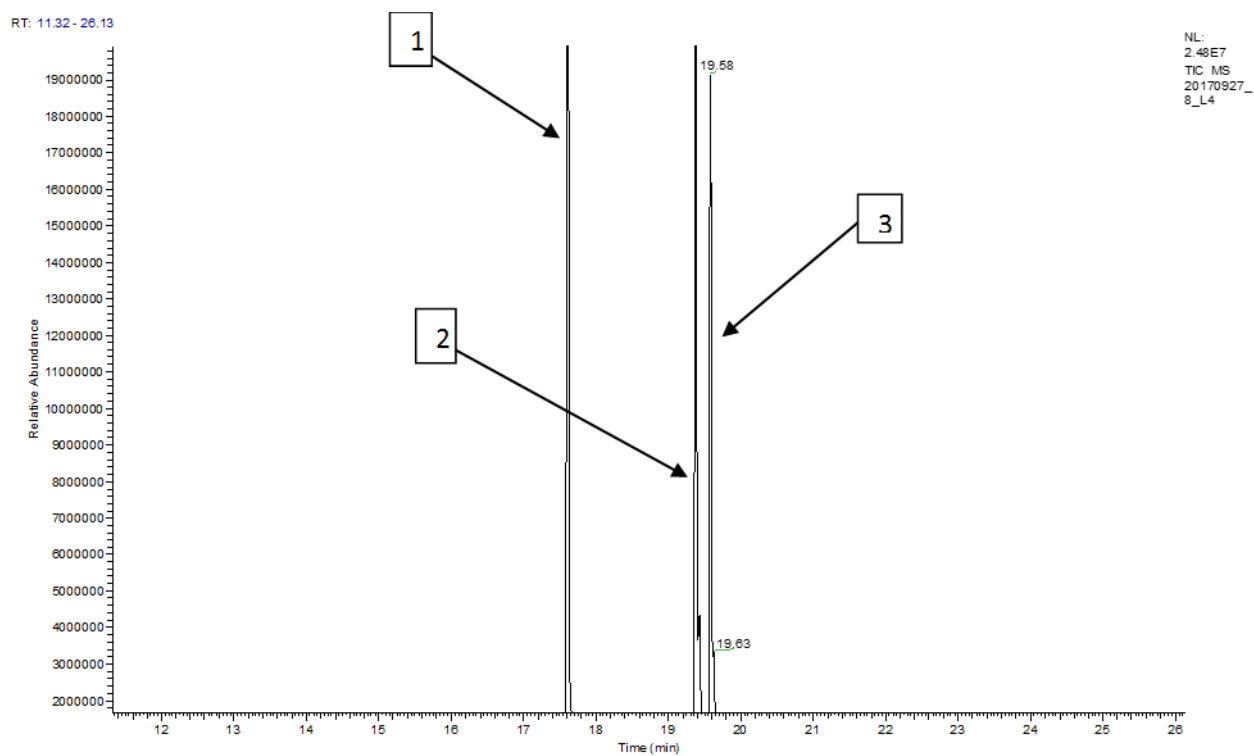


Figure 22: GC chromatogram of fraction L4 from the leaf extract of *T. lupinifolia*.

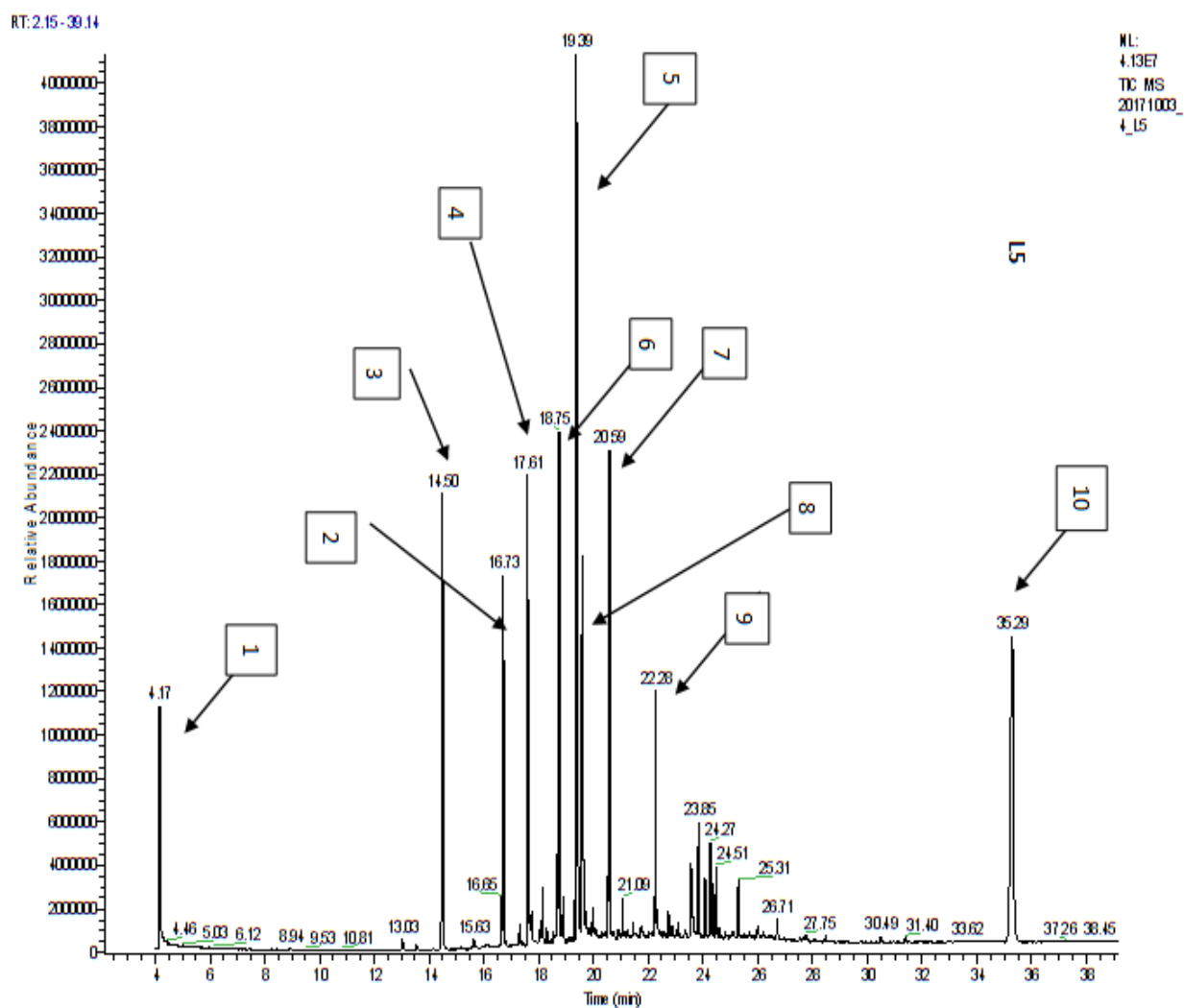


Figure 23: GC chromatogram of fraction L5 from the leaf extract of *T. lupinifolia*.

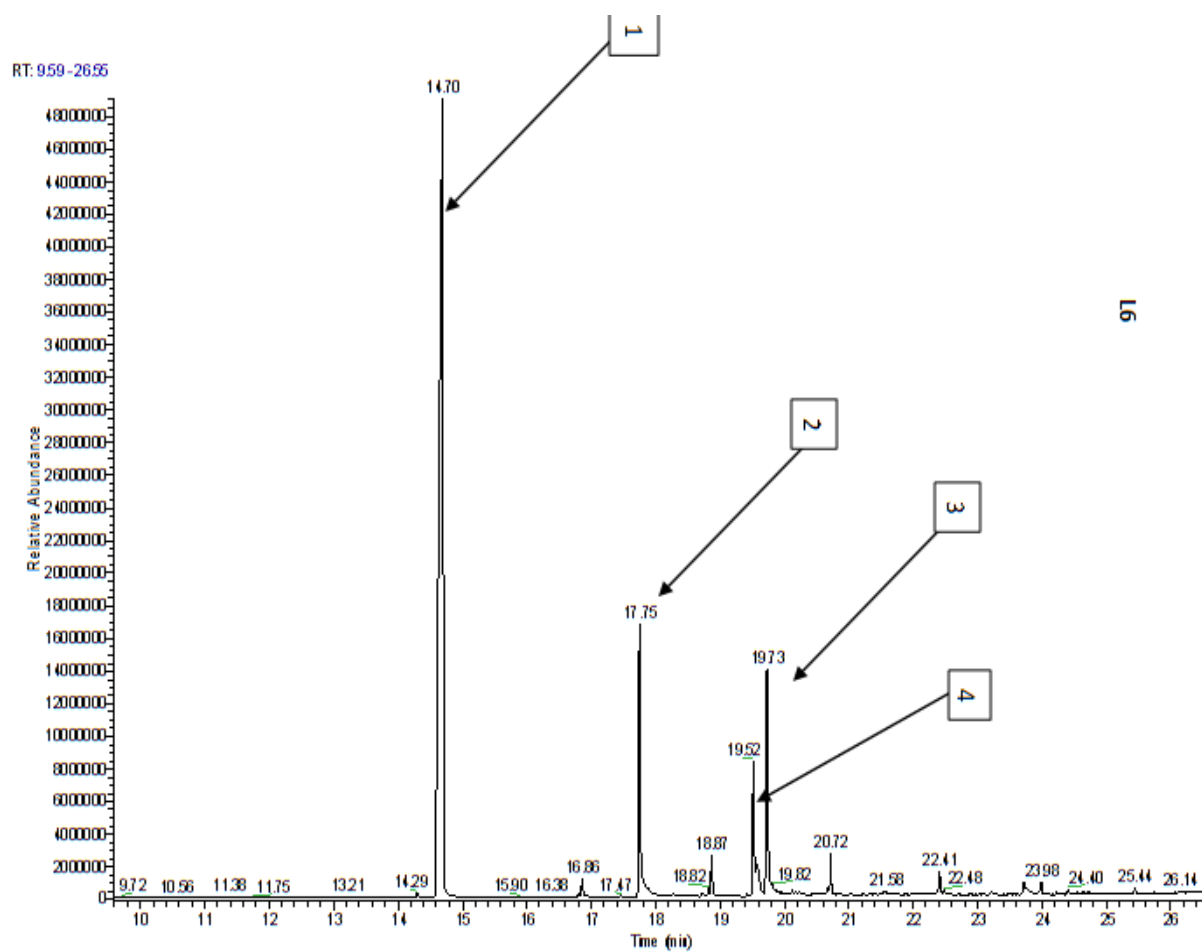


Figure 24: GC chromatogram of fraction L6 from the leaf extract of *T. lupinifolia*.

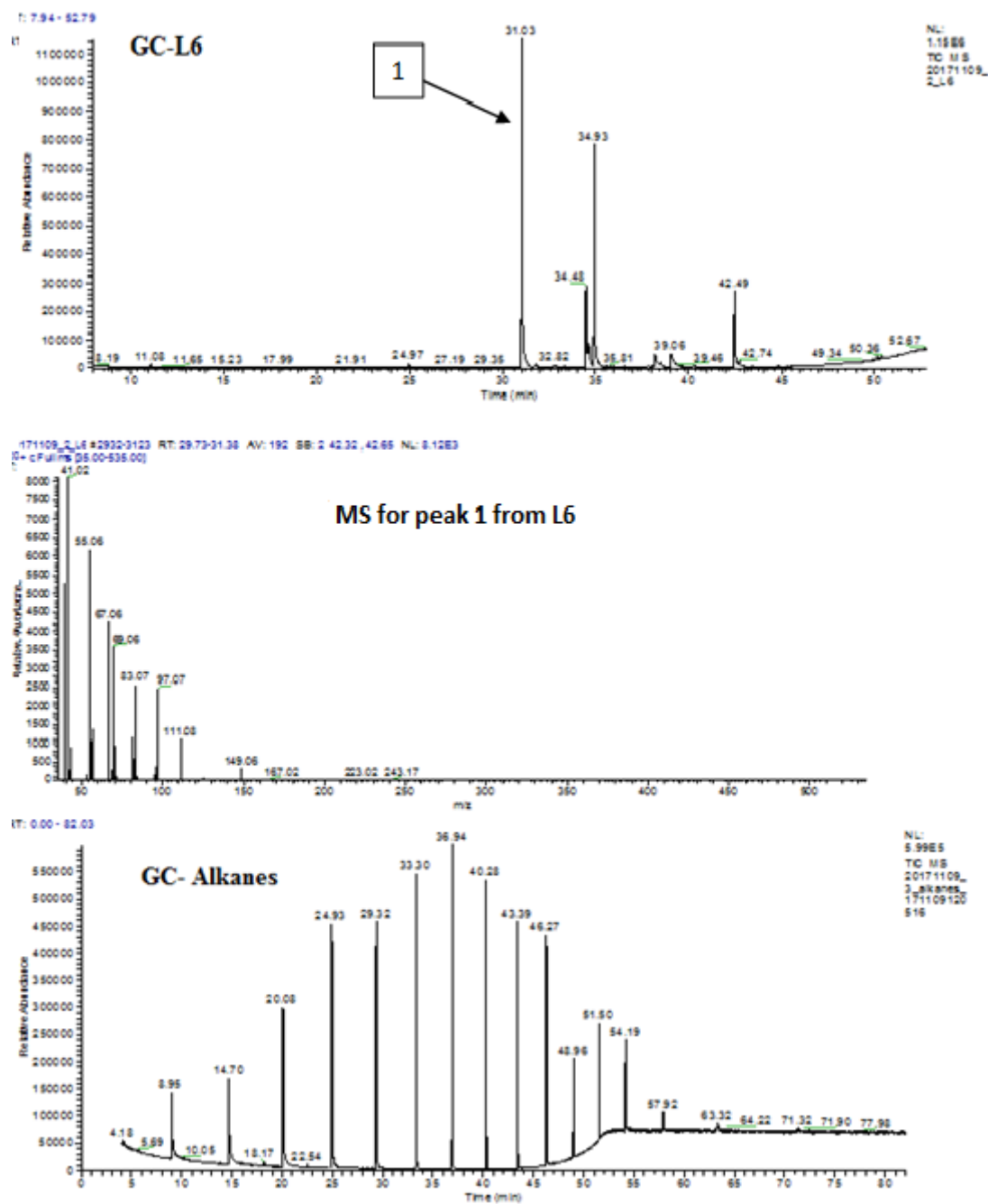


Figure 25: GC chromatogram of the fraction L6 and the standard alkane mixture from the leaf extract of *T. lupinifolia*.



MINISTRY OF ENVIRONMENT AND TOURISM

RESEARCH/COLLECTING PERMIT

Permit Number 2223/2016

Valid from 1 January 2017 to 31 December 2017

Permission is hereby granted in terms of the Nature Conservation Ordinance 1975 (Ord. 4 of 1975) to:

Name: **D. Nanhapo**
Address: **Department of Chemistry and Biochemistry**
University of Namibia
Private Bag 13301
Windhoek
Namibia

Coworkers: **Dr. H. Renate and Prof. K. Koch**

Isolation and characterization of major metabolites from roots of *Tephrosia lupinifolia* and their utilization as templates in synthesis of potential anti-infective agents at Tsumkwe (Otjozondjupa region) and surrounding areas, subject to attached conditions.

IMPORTANT: This permit is not valid if altered in any way.





Authorising Officer

IMPORTANT

This permit is subject to the provisions of the Nature Conservation Ordinance, 1975 (Ordinance 4 of 1975) and the regulations promulgated thereunder, and the holder is subject to all such conditions and regulations.

Enquiries: Conservation Scientist, email DL@DENVNMBOT13301.NA
Private Bag 13301, Windhoek, Namibia

Figure 26: Research/collection permit



ETHICAL CLEARANCE CERTIFICATE

Ethical Clearance Reference Number: FOS/136/2016

Date: 5 December, 2016

This Ethical Clearance Certificate is issued by the University of Namibia Research Ethics Committee (UREC) in accordance with the University of Namibia's Research Ethics Policy and Guidelines. Ethical approval is given in respect of undertakings contained in the Research Project outlined below. This Certificate is issued on the recommendations of the ethical evaluation done by the Faculty/Centre/Campus Research & Publications Committee sitting with the Postgraduate Studies Committee.

Title of Project: Isolation and Characterisation of Major Metabolites From The Roots of *Tephrosia Lupinifolia* and their Utilisation as Templates In The Synthesis of Potential Anti-Infective Agents

Nature/Level of Project: Masters

Researcher: N. David

Student Number: 201092557

Faculty: Faculty of Science

Supervisors: Dr. R. Hans (Main) Prof. K. Koch (Co)

Take note of the following:

- (a) Any significant changes in the conditions or undertakings outlined in the approved Proposal must be communicated to the UREC. An application to make amendments may be necessary.
- (b) Any breaches of ethical undertakings or practices that have an impact on ethical conduct of the research must be reported to the UREC.
- (c) The Principal Researcher must report issues of ethical compliance to the UREC (through the Chairperson of the Faculty/Centre/Campus Research & Publications Committee) at the end of the Project or as may be requested by UREC.
- (d) The UREC retains the right to:
 - (i) Withdraw or amend this Ethical Clearance if any unethical practices (as outlined in the Research Ethics Policy) have been detected or suspected,
 - (ii) Request for an ethical compliance report at any point during the course of the research.

UREC wishes you the best in your research.

Prof. P. Odonkor: UREC Chairperson

A handwritten signature in black ink, appearing to be "P. Odonkor", written over a horizontal line.

Ms. P. Claassen: UREC Secretary

A handwritten signature in black ink, appearing to be "P. Claassen", written over a horizontal line.

Figure 27: Ethical clearance certificate