FACULTY OF SCIENCE AND EDUCATION

DEPARTMENT OF CHEMISTRY

TLC-BIOAUTOGRAPHY GUIDED IDENTIFICATION AND GC-MS CHARACTERIZATION OF ANTIBACTERIAL COMPOUNDS FROM CANNABIS SATIVA LEAVES

BY

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DECLARATION

I, **Obuni Jude**, declare that this research project is my original work and has not been submitted to any other institution for academic credit. All sources of information used have been duly acknowledged.

Signature: 

Date: 15th/09/2026

APPROVAL

This research project has been submitted with the approval of the supervisor.

Supervisor's Name: **Dr. Andima Moses (PhD)**

Signature.....

Date.....15/2/2026.....

DEDICATION

I dedicate this research project to the Almighty God for His grace, strength and guidance throughout my academic journey. I also dedicate this work to my parents, family members and all those who supported and encouraged me throughout my undergraduate studies. Finally, I dedicate this research to students, researchers and communities interested in studying more about Cannabis sativa.

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ABSTRACT

The emergence of antibiotic-resistant bacteria has created an urgent need for novel antibacterial agents from natural sources. This study aimed to localize, recover and tentatively characterize antibacterial constituents from Cannabis sativa leaves using TLC-bioautography-guided isolation followed by GC-MS analysis. Fresh Cannabis sativa leaves were collected, dried and extracted by maceration using three solvents of increasing polarity, namely hexane, ethyl acetate and ethanol. The crude extracts were screened for antibacterial activity against Staphylococcus aureus (Gram-positive) and Escherichia coli (Gram-negative) using direct TLC-bioautography with resazurin dye as a microbial-growth indicator.

The extracts produced distinct inhibition zones (blue or colourless regions against a pink background) against both bacterial strains, with a visually more pronounced response observed for S. aureus, consistent with the absence of an outer membrane in Gram-positive bacteria; this observation is descriptive rather than quantitative, as inhibition-zone measurements and replicate testing were not performed. Ethyl acetate produced four cleanly separated spots with no co-elution under the hexane:ethyl acetate (7:3) TLC mobile-phase system used, indicating that the ethyl acetate extract yielded the cleanest chromatographic profile among the three extracts. Ethanol produced six spots with co-elution at a retardation factor (Rf) of 0.4, while hexane produced five spots with co-elution at Rf 0.5.

GC-MS analysis of the recovered active bands from the ethanol extract generated tentative, library-based annotations for several constituents, including a compound provisionally annotated as a Cannflavin-related flavonoid (consistent with 2',4'-dimethoxy-3-hydroxy-6-methylflavone) from Spot 2 (Rf 0.5), a compound provisionally annotated as oleanolic acid from Spot 3 (Rf 0.4), and fatty-acid esters including methyl palmitate, methyl stearate and methyl oleate. The hexane and ethyl acetate extracts were associated primarily with putative alkanes (eicosane, heneicosane), fatty-acid esters and sterol-related compounds. Because these assignments rest on GC-MS spectral-library matching without confirmation by authentic standards, retention-index comparison, or complementary spectroscopy, and because no minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) or dose-response data were generated for isolated compounds, all compound identities and potency comparisons reported here should be regarded as tentative rather than confirmed.

Within this limitation, the study indicates that Cannabis sativa leaf extracts contain multiple chromatographically separable constituents associated with antibacterial activity, with the ethanol extract yielding the constituents of greatest apparent interest despite co-elution, and the ethyl acetate extract offering the cleanest analytical separation. These findings provide preliminary, exploratory support for the traditional use of Cannabis sativa in managing bacterial infections and identify candidate bands — most notably the putative Cannflavin-related flavonoid — that warrant purification, structural confirmation and quantitative antibacterial testing in future work.

LIST OF ABBREVIATIONS

AMR – Antimicrobial Resistance

CBD – Cannabidiol

E. coli – Escherichia coli

ESBL – Extended-Spectrum Beta-Lactamase

GC-MS – Gas Chromatography–Mass Spectrometry

MRSA – Methicillin-Resistant Staphylococcus aureus

S. aureus – Staphylococcus aureus

THC – Tetrahydrocannabinol

TLC – Thin Layer Chromatography

Rf – Retardation Factor

MIC – Minimum Inhibitory Concentration

MBC – Minimum Bactericidal Concentration

CHAPTER ONE: INTRODUCTION

1.1 Background of the Study

Antimicrobial resistance (AMR) is one of the greatest threats to modern medicine, with infections that were once easily treatable now becoming life-threatening (Avoseh et al., 2025). The urgent need for new antibacterial agents has shifted attention towards natural products, especially those derived from plants, particularly Cannabis sativa. Cannabis sativa, commonly known for its psychoactive cannabinoids such as THC and CBD, also produces a wide range of secondary metabolites including flavonoids, terpenes and phenolic compounds (Cannabinoids, 2007). These compounds are part of the plant's natural defence system and may hold untapped potential as antibacterial agents.

Medicinal plants have long been used for treating various infections, and traditional communities have relied on them for generations. According to the World Health Organization, approximately 80 percent of the population in Africa uses herbal medicine to treat and manage diseases; medicinal plants remain indispensable because they are readily available and culturally accepted.

Among the medicinal plant families, Cannabaceae contains many species possessing pharmacologically important compounds (ElSohly et al., 2017). The genus Cannabis comprises several hundred natural compounds and is distributed across Asia, Australia and parts of Africa. In several African communities, Cannabis species are used to help manage skin irritation, stomach problems, skin infections, wounds, fever, cough, inflammation, microbial infections and nausea, owing to their reported antibacterial, antioxidant, anti-inflammatory and antifungal properties (Avoseh et al., 2025).

Cannabis sativa is a medicinal plant belonging to the family Cannabaceae that is widely distributed and cultivated in some regions; the plant is used locally by traditional healers for the management of several diseases and infections. Previous phytochemical studies indicate that Cannabis sativa contains flavonoids, phenolic compounds, steroids and essential oils, several of which possess antibacterial properties, with reported traditional applications for skin irritation, digestive problems, nausea and other ailments. There is an urgent need for new antibacterial agents because

many existing antibiotics are becoming less effective, largely as a result of increasing bacterial resistance. This growing antimicrobial resistance (AMR) has become one of the major global public health challenges (Ferri et al., 2017). Pathogenic bacteria such as Escherichia coli and Staphylococcus aureus have developed resistance to many commonly prescribed antibiotics, making treatment of bacterial infections increasingly difficult and creating an urgent need to discover new antibacterial compounds that can serve as alternatives to existing antibiotics.

Plant extracts contain hundreds of phytochemicals with varying biological activities (Dagdag et al., 2024). Conventional isolation of bioactive compounds from crude plant extracts is often expensive and time-consuming, because inactive compounds are purified together with active compounds (Rajabiyan et al., 2025). Bioautography offers an efficient alternative for the detection of antibacterial compounds: it is comparatively fast and allows localization of activity even within a complex matrix. When performed with thin-layer chromatographic adsorbents, bioautography can be used to assess antibacterial activity directly, since compounds are first separated on a TLC plate before antibacterial activity is assessed directly on the chromatogram (Móricz et al., 2019). The method enables rapid localization of antibacterial compounds without requiring complete purification of every extracted constituent, thereby reducing the time and resources required during early-stage drug discovery (Czernicka et al., 2020).

Direct TLC-bioautography is particularly useful for locating active compounds within a complex mixture. Following chromatographic separation, bacterial suspensions are applied directly onto the developed TLC plate (Czernicka et al., 2020). During incubation, antibacterial compounds inhibit bacterial growth at their respective positions. After spraying with resazurin dye, viable bacteria reduce the blue dye to pink, whereas regions containing antibacterial compounds remain colourless or blue, producing inhibition zones that correspond to the positions of active constituents. This makes the bioassay-guided isolation process considerably more tractable, particularly for identifying active compounds within complex plant extracts.

1.2 Statement of the Problem

Despite the documented traditional use of Cannabis sativa in managing bacterial infections, the specific phytochemical constituents responsible for antibacterial activity against clinically significant pathogens such as Staphylococcus aureus and Escherichia coli remain inadequately

characterized. Current evidence relies largely on crude-extract studies without identification of individual bioactive compounds. Furthermore, the emergence of multidrug-resistant strains (such as MRSA and ESBL-producing E. coli) demands novel antibacterial agents, yet no standardized protocol exists for the bioassay-guided isolation of Cannabis sativa's antibacterial principles. This study addresses this gap by employing TLC-bioautography — a technique that enables targeted, activity-guided isolation of active compounds from complex plant matrices — followed by GC-MS analysis to generate tentative chemical annotations of the recovered antibacterial constituents.

1.3 Purpose of the Study

The purpose of this study was to localize and tentatively characterize antibacterial constituents from Cannabis sativa using a TLC-bioautography-guided approach. This involved the extraction, chromatographic separation and recovery of active fractions, followed by GC-MS-based chemical annotation and a preliminary evaluation of activity patterns against relevant bacterial strains.

1.4 Objectives

1.4.1 General Objective

To identify and characterize antibacterial compound(s) from Cannabis sativa leaves using TLC-bioautography-guided isolation and to evaluate their antibacterial spectrum against Escherichia coli and Staphylococcus aureus.

1.4.2 Specific Objectives

i. To prepare crude extracts of Cannabis sativa using solvents of varying polarity.

ii. To screen the crude extracts for antibacterial activity using an agar diffusion assay.

iii. To develop and apply a direct TLC-bioautography procedure to screen Cannabis sativa extracts for antibacterial activity against the selected bacterial strains.

iv. To facilitate the tentative identification of active compounds using gas chromatography-mass spectrometry (GC-MS).

1.5 Research Questions

i. Does the crude extract of Cannabis sativa contain antibacterial compounds?

ii. Is the extract effective against Escherichia coli and Staphylococcus aureus?

iii. Which chromatographic fractions exhibit antibacterial activity following TLC-bioautography?

iv. Which phytochemical compounds are tentatively associated with the observed antibacterial activity, and what does the available evidence suggest about the antibacterial potency of the isolated bands?

1.6 Scope of the Study

This study focused on the leaves of Cannabis sativa and was designed to remain focused, achievable and meaningful within the resources available. It defines the plant material, methods, bacterial strains and parameters investigated.

The antibacterial properties of Cannabis sativa were evaluated against two bacteria commonly associated with human infections, one Gram-positive and one Gram-negative. Compounds were localized and tentatively characterized using TLC-bioautography and GC-MS, with resazurin dye used as the microbial-growth indicator. Extraction was performed by maceration using solvents of increasing polarity. Confirmatory techniques such as MIC/MBC determination, structural elucidation by NMR, and testing against clinically characterized multidrug-resistant strains fall outside the scope of this study and are identified as priorities for future work.

1.7 Significance of the Study

i. The study contributes preliminary evidence toward the discovery of alternative antibacterial agents from a locally available plant.

ii. It provides exploratory support for the traditional use of Cannabis sativa in managing bacterial infections.

iii. The study generates tentative chemical annotations for candidate antibacterial constituents in Cannabis sativa that can guide future purification and confirmatory work.

iv. The study provides baseline chromatographic and GC-MS data for future isolation and structural characterization of active compounds.

CHAPTER TWO: LITERATURE REVIEW

2.1 Introduction

This chapter reviews literature relevant to antimicrobial resistance, medicinal plants in antibacterial drug discovery, the phytochemistry and reported antibacterial activity of Cannabis sativa, the two test organisms used in this study, thin-layer chromatography (TLC), TLC-bioautography, resazurin-based viability detection, and gas chromatography-mass spectrometry (GC-MS) (Fordjour et al., 2023). The review provides the scientific background for using bioautography-guided isolation and GC-MS-based annotation of antibacterial compounds from Cannabis sativa, and closes by identifying the specific knowledge gap this study addresses.

2.2 Antimicrobial Resistance

Antimicrobial resistance is a biochemical phenomenon in which bacteria evolve mechanisms to survive exposure to drugs that once killed them. This occurs through gene mutation, horizontal gene transfer, or selective pressure arising from the overuse of antibiotics. Misuse and overuse of antibiotics have accelerated the emergence of resistant bacterial strains and AMR has become a global public health crisis (English et al., 2010). Among the bacteria showing increasing resistance are Staphylococcus aureus and Escherichia coli; resistance in these organisms has developed through alteration of antibiotic targets, reduced membrane permeability, efflux-pump mechanisms and enzymatic drug inactivation. Increasing resistance raises the cost of treatment, motivating an active search for alternative antibacterial compounds from medicinal plants (English et al., 2010).

2.3 Medicinal Plants in Antibacterial Drug Discovery

Medicinal plants still serve as the primary source of healthcare for a large share of the world's population, particularly in low- and middle-income settings, and remain an important reservoir for the discovery of new antibacterial scaffolds. Plant extracts contain hundreds of phytochemicals with varying biological activities, and activity-guided fractionation approaches — rather than crude-extract screening alone — are increasingly used to localize and prioritize the specific constituents responsible for observed bioactivity (Dagdag et al., 2024; Rajabiyan et al., 2025).

2.4 Cannabis sativa: Taxonomy and Medicinal Relevance

The genus Cannabis, belonging to the family Cannabaceae, is distributed across Asia, Africa and Australia (Research, 2018). Many species within this family are used traditionally for the treatment of skin infections, infectious diseases and respiratory conditions; medicinal plants remain the primary healthcare resource for a substantial proportion of the African population.

Cannabis sativa is commonly used by traditional practitioners to manage cough, skin conditions, infected wounds, malaria, respiratory conditions and stomach upset (Shakil et al., 2021). Use of the plant is frequently motivated by the cost of imported antibiotics and by increasing antibiotic resistance. Plant extracts, particularly the volatile essential oil obtained from the leaves, have been reported to exhibit antioxidant, antibacterial, anti-inflammatory and fungitoxic properties, although the composition and activity of the essential oil vary with geographical region and variety. This variability makes it important to examine indigenous Cannabis sativa populations to evaluate their possible antibacterial properties in the local context.

According to clinical epidemiological reports, Staphylococcus aureus and Pseudomonas aeruginosa are among the predominant drug-resistant organisms implicated in nosocomial infections, with infection rates reported as high as 50% in some settings. The presence of these drug-resistant strains complicates treatment and drives the search for alternative antibacterial agents. Phytochemical studies of Cannabis sativa have identified several bioactive classes, including phenolic acids, flavonoids and essential-oil components such as terpenoids, steroids, tannins and saponins, several of which are reported to possess antibacterial properties (Rock et al., 2021).

2.5 Phytochemistry of Cannabis sativa

Cannabis sativa contains a range of biologically active phytochemical classes, summarized below.

Flavonoids

Flavonoids are polyphenolic compounds possessing a C6-C3-C6 backbone. They are reported to insert into bacterial membranes, chelate metal ions, and interfere with DNA replication (Xue et al., 2025).

Phenolic compounds

Phenolic compounds are reported to damage bacterial cell walls and interfere with essential metabolic pathways; several also have antioxidant properties that reduce oxidative damage (Bhattacharya et al., 2025).

Terpenes

Terpenes are built from isoprene units (C5H8); they are hydrophobic and are reported to disrupt bacterial membranes by increasing permeability (Evolution, 2024).

Tannins

Tannins are large polyphenols that precipitate proteins, thereby inactivating bacterial enzymes (Chung et al., 1998).

Saponins

Saponins increase membrane permeability and are reported to inhibit bacterial growth.

Essential oils

Essential oils obtained from Cannabis species contain volatile compounds reported to be responsible for antibacterial, antioxidant and anti-inflammatory activities.

These phytochemical classes are proposed to act synergistically to produce the medicinal effects observed for Cannabis sativa (Naz et al., 2023); however, it should be noted that not all of these constituents — particularly larger or thermally labile compounds — are equally amenable to detection by GC-MS, a point revisited in Section 2.11.

2.6 Reported Antibacterial Activity of Cannabis sativa

Various studies report that extracts obtained from Cannabis sativa possess antibacterial activity against both Gram-positive and Gram-negative bacteria. Leaf extracts have been reported to inhibit Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Bacillus subtilis. The extent of antibacterial activity is influenced by the geographical location of the plant, the solvent used for extraction, and the maturity of the leaves (Majhi et al., 2023).

2.7 Staphylococcus aureus and Escherichia coli as Test Organisms

Escherichia coli

Escherichia coli is a Gram-negative, rod-shaped bacterium belonging to the family Enterobacteriaceae. Although most strains inhabit the human intestine as commensals, pathogenic strains can cause urinary tract infections, diarrhoea and septicaemia (Podschun et al., 1998). E. coli exhibits comparatively high intrinsic resistance to many antibiotics because of its outer membrane, which acts as a permeability barrier (Chemotherapy, 1989).

Staphylococcus aureus

Staphylococcus aureus is a Gram-positive coccus arranged in grape-like clusters that can cause wound infections, pneumonia, skin infection, septicaemia and food poisoning. Some strains, notably methicillin-resistant Staphylococcus aureus (MRSA), are resistant to multiple antibiotics (Liu et al., 2021).

2.8 Thin-Layer Chromatography (TLC) in Natural-Products Analysis

TLC is one of the most widely used techniques for the preliminary separation of phytochemicals and involves a stationary phase (silica gel) and a mobile phase (solvent system) (Liu et al., 2020). Different compounds migrate different distances depending on their polarity: highly polar compounds remain closer to the origin, while less polar compounds move further with the solvent front (Techniques, 2014).

2.9 TLC-Bioautography

Bioautography is regarded as one of the most useful assays for detecting antibacterial compounds because it allows localization of activity even within a complex matrix, permitting target-directed isolation of active constituents. Bioautography assays are generally categorized into three groups:

i. Direct bioautography, in which microorganisms grow directly on the developed TLC plate (Jesionek et al., 2013).

ii. Contact bioautography, in which antibacterial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact (Hamburger & Cordell, 1987).

iii. Agar overlay (immersion) bioautography, in which a seeded agar medium is applied onto the TLC plate; this method can be considered a hybrid of direct and contact bioautography. In contact bioautography specifically, the transfer process tends to produce larger inhibition zones, which can reduce sensitivity and the ability to discriminate between active compounds with similar Rf values (Rahalison et al., 1991).

Direct TLC-bioautography is an effect-directed analysis technique that enables the search for bioactive substances within complex mixtures such as plant extracts. It offers a simple, low-cost approach with useful sensitivity and specificity for localizing, and subsequently evaluating, constituents of interest, and is frequently used as a fast pre-screen that narrows down the fractions requiring more advanced analysis. Recent literature illustrates its use for identifying antibacterial compounds from plant extracts and essential oils, with subsequent GC-MS identification of the active fractions (Rahalison et al., 1991).

2.10 Resazurin Viability Detection

Resazurin dye is widely used to visualize bacterial growth on TLC plates. Living bacteria reduce blue resazurin to pink resorufin, whereas in zones where antibacterial compounds inhibit growth, the dye is not reduced (Palmieri et al., 2026). Consequently, clear or blue-coloured inhibition zones on the TLC plate correspond to the positions of antibacterial compounds.

2.11 GC-MS Characterization: Strengths and Limitations

Following localization of antibacterial bands, GC-MS is commonly employed for tentative compound annotation, using retention behaviour, molecular weight, mass-to-charge ratio and fragmentation pattern compared against reference spectral libraries (Palmieri et al., 2026). GC-MS is widely used in natural-products chemistry because it is sensitive and can resolve complex mixtures of volatile and semi-volatile constituents. However, GC-MS is not equally suited to all classes of phytochemical: larger, thermally labile, highly polar or non-volatile compounds may require derivatization, may not elute cleanly, or may be poorly represented in general-purpose spectral libraries. A library match alone — without confirmation by authentic standards, retention-index comparison, or complementary techniques such as LC-MS/MS or NMR — constitutes a

tentative annotation rather than a confirmed structural identification. This distinction is applied throughout the results and discussion in Chapter Four.

2.12 Bioassay-Guided Fractionation

Bioassay-guided fractionation combines chromatographic separation with biological testing at each stage, so that only fractions showing activity are advanced for further purification and characterization. This reduces the resources required relative to exhaustive phytochemical profiling of a crude extract and is the general strategy adopted in this study, in which TLC-bioautography was used to localize active bands prior to their recovery and GC-MS analysis.

2.13 Knowledge Gap

Although Cannabis sativa has demonstrated antibacterial activity in several studies, limited information exists regarding the specific compounds responsible for this activity (Appendino et al., 2008). Most published studies focus on crude extracts rather than bioassay-guided isolation of individual antibacterial constituents, and few studies combine TLC-bioautography with GC-MS for tentative characterization of antibacterial compounds from Cannabis sativa. This study seeks to contribute toward bridging this gap by employing direct TLC-bioautography-guided isolation followed by GC-MS-based tentative identification of antibacterial constituents from different Cannabis sativa leaf extracts, while explicitly flagging the confirmatory work still required before these constituents can be regarded as established antibacterial agents.

CHAPTER THREE: METHODOLOGY

3.1 Introduction

This chapter describes the procedures followed in the identification of antibacterial compounds from Cannabis sativa using TLC-bioautography-guided isolation and GC-MS analysis.

3.2 Study Design

The study used an experimental, laboratory-based design focused on bioassay-guided isolation. Plant extracts were separated into chromatographic fractions, each of which was tested for antibacterial activity; only the active fractions were advanced for recovery and GC-MS analysis. The study analysed the plant extracts for phytochemicals that could plausibly be associated with the observed antibacterial activity.

3.3 Study Area

The study was conducted in the Chemistry and Biology laboratories of Busitema University, Nagongera Campus, Tororo District, Eastern Uganda. Plant material was collected from Nagongera village, where the Cannabis sativa plants used were cultivated and monitored by local individuals.

3.4 Plant Collection, Authorization and Authentication

Because Cannabis sativa is a regulated plant in many jurisdictions, plant material for this study was collected under the supervision of the Department of Chemistry, and use of the material was confined to the University's teaching and research laboratories in accordance with institutional research procedures.

Fresh, healthy leaves of Cannabis sativa were collected during the dry season to minimize moisture content. The plant specimen was authenticated by departmental staff prior to use.

3.5 Study Materials

Plant material

Fresh Cannabis sativa leaves, collected and authenticated as described above.

Test microorganisms

Antibacterial activity was evaluated against two laboratory reference bacterial cultures: a Gram-positive bacterium (Staphylococcus aureus) and a Gram-negative bacterium (Escherichia coli). These organisms were selected because they represent both major Gram-staining categories and are among the most common pathogens implicated in human bacterial infections. Detailed strain designation (e.g., ATCC number), clinical source and antibiotic-susceptibility profile were not available for the isolates used and are noted as a limitation in Section 4.10.

3.6 Apparatus and Equipment

Analytical balance, electrical grinder, hot-air oven, incubator, autoclave, glass capillary tubes, measuring cylinder, glass beakers, conical flasks, volumetric flasks, sterile test tubes, TLC developing chamber, UV lamp, TLC silica gel 60 F254 plates, GC-MS instrument, forceps, sterile inoculating loops.

3.7 Chemicals and Reagents

Cannabis sativa extract, ethanol, hexane, ethyl acetate, resazurin dye, distilled water, nutrient agar (NA), MacConkey agar, sterile water, and hexane:ethyl acetate (7:3) as the TLC mobile-phase (solvent) system.

3.8 Procedures

3.8.1 Plant material preparation

Fresh leaves of Cannabis sativa were collected from Nagongera village and authenticated. Leaves were washed with distilled water, spread on a clean tray and dried under shade at room temperature to preserve phytochemicals. The dried leaves were ground into a fine powder using an electrical grinder, and the powdered sample was stored in airtight containers until extraction.

3.8.2 Extraction by maceration

5 g of powdered Cannabis sativa was weighed and soaked in 50 mL of solvent of varying polarity (hexane, ethanol, ethyl acetate, respectively) in a glass beaker, which was covered to minimize evaporation and contamination. The mixture was kept at room temperature and left to stand for 72 hours with occasional shaking. Each extract was filtered using Whatman filter paper and the filtrate was stored until TLC analysis.

3.8.3 Preparation of culture media

MacConkey agar for Escherichia coli: 52 g of MacConkey agar powder was dissolved in one litre of distilled water. The medium was heated with stirring until fully dissolved and then sterilized by autoclaving at 120°C for 15 minutes. After sterilization, the medium was cooled to approximately 50°C, and approximately 20 mL was poured into sterile Petri dishes, which were allowed to cool and solidify before inoculation. MacConkey agar was selected because it is selective for Gram-negative bacteria and differentiates lactose fermenters such as Escherichia coli.

Nutrient agar for Staphylococcus aureus: 28 g of nutrient agar powder was dissolved in one litre of distilled water, heated with stirring until fully dissolved, and sterilized by autoclaving at 120°C for 15 minutes. After sterilization, the medium was cooled to approximately 50°C, and approximately 20 mL was poured into sterile Petri dishes, which were allowed to cool and solidify before inoculation. Nutrient agar was selected because it supports the growth of Staphylococcus aureus without inhibiting bacterial metabolism.

3.8.4 Preparation of bacterial inoculum

Pure bacterial colonies grown for 24 hours were selected, and several colonies were transferred into sterile normal saline, with the bacterial concentration adjusted to approximately 1.5 × 10^8 cells/mL by matching against the 0.5 McFarland turbidity standard.

3.8.5 Thin-layer chromatography (TLC)

Silica gel 60 F254 TLC plates were used, and extracts were applied to the plates using glass capillary tubes. Plates were developed in a hexane:ethyl acetate (7:3) mobile-phase system. Separated spots were visualized under UV light at 254 nm and 366 nm, and Rf values were calculated for each separated compound. Plate dimensions, exact sample application volume/concentration, and chamber saturation conditions were not precisely recorded in this study

and are recommended for detailed documentation in future replications so that the chromatographic method is fully reproducible.

3.8.6 Direct TLC-bioautography

Test organisms: Escherichia coli and Staphylococcus aureus.

Developed TLC plates were sterilized (e.g., under UV light) and then sprayed evenly with a bacterial suspension, after which they were incubated at 37°C for 24 hours. During incubation, bacterial growth occurred uniformly across the chromatogram except at positions where antibacterial compounds were present, which inhibited bacterial growth.

3.8.8 Visualization of antibacterial activity using resazurin dye

After incubation, antibacterial activity was visualized by spraying resazurin dye evenly over each TLC plate, which was then returned to the incubator for approximately 40 minutes at 37°C. During this period, living bacterial cells reduced the blue resazurin dye to pink, while areas containing antibacterial compounds remained blue or colourless because bacterial growth had been inhibited at those positions.

3.8.9 Recovery of active compounds

The silica gel corresponding to each active band was carefully scraped from the TLC plate using a sterile blade and transferred into a sterile test tube. Approximately 5 mL of ethanol was added to each tube, and the mixture was allowed to stand for 15 minutes to dissolve the adsorbed compounds. The suspension was filtered, and the filtrate was placed in a sterile glass vial and allowed to evaporate; the resulting residues were stored pending GC-MS analysis.

3.8.10 GC-MS analysis

Recovered residues were analysed by gas chromatography-mass spectrometry to generate mass spectra for tentative compound annotation. Detailed instrument operating parameters — including the instrument model, column type and dimensions, carrier gas and flow rate, injection mode and volume, injector and oven-temperature programme, ionization mode and energy, ion-source temperature, mass scan range, solvent delay, and the specific spectral library and match-score cut-off used — were not comprehensively recorded during data collection for this project.

3.8.11 Compound-identification criteria used in this report

In the absence of authentic reference standards, retention-index confirmation or complementary spectroscopic techniques, compounds discussed in this report are described as "tentatively annotated", "putatively identified" or "provisionally assigned" based on GC-MS spectral-library comparison alone. This wording is used consistently in Chapter Four and Chapter Five, replacing the more definitive language (e.g., "identified") used in earlier drafts of this work, in order to accurately represent the level of certainty the data support.

3.8.12 Data analysis

Rf values were calculated for each TLC spot as the ratio of the distance travelled by the compound to the distance travelled by the solvent front. GC-MS peak areas, retention times, and library-suggested molecular formulae and weights were tabulated for each recovered band. Because each extract and bioautography assay was analysed once rather than in replicate, descriptive reporting (tables and figures) rather than inferential statistical analysis was used to present the results; replicate testing is recommended for future work so that variability can be quantified.

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Extraction

Three extracts of Cannabis sativa leaves were prepared by 72-hour maceration in hexane, ethyl acetate and ethanol, respectively, as described in Section 3.8.2. As noted in that section, the dry mass of each crude extract was not determined, so extraction yield could not be calculated or compared quantitatively across solvents. The comparisons that follow are therefore based on the chromatographic and GC-MS profile of each extract rather than on the quantity of material extracted.

4.2 Antibacterial Activity and TLC-Bioautography

The TLC-bioautography assay indicated that Cannabis sativa extracts possess antibacterial activity against both Staphylococcus aureus and Escherichia coli. Inhibition zones observed on TLC plates after spraying with resazurin dye were consistent with the presence of antibacterial compounds: blue or colourless zones against a pink background indicated that bacterial growth was inhibited at specific Rf positions. A visually more pronounced response was observed against S. aureus compared with E. coli, which is consistent with known structural differences between Gram-positive and Gram-negative bacteria — S. aureus lacks an outer membrane and is generally more susceptible to lipophilic compounds such as cannabinoids and terpenes, whereas the outer membrane of E. coli acts as an additional permeability barrier that can reduce the efficacy of some compounds.

4.3 Putatively Annotated Compounds — Ethanol Extract

Table 1 presents the Rf values obtained for the six spots separated from the ethanol extract using the hexane:ethyl acetate (7:3) TLC mobile phase.

Table 1: Retardation factor for the separation achieved using ethanol (solvent front = 7.0 cm)

Spot number	Distance moved by compound (cm)	Retardation factor (Rf)
1	4.3	0.6
2	3.7	0.5
3	3.0	0.4
4	2.5	0.4
5	2.0	0.3
6	1.5	0.2

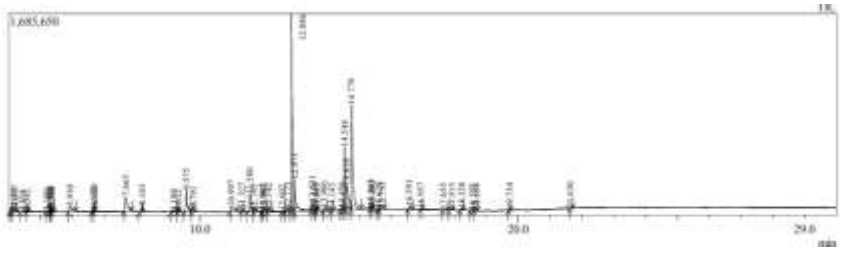

Figure 1: Peaks for Spot 1 of the ethanol extract

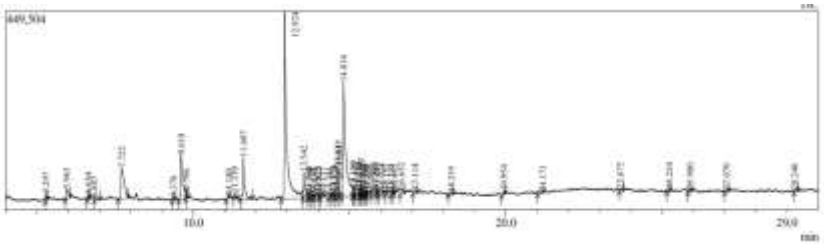

Figure 2: Peaks for Spot 2 of the ethanol extract

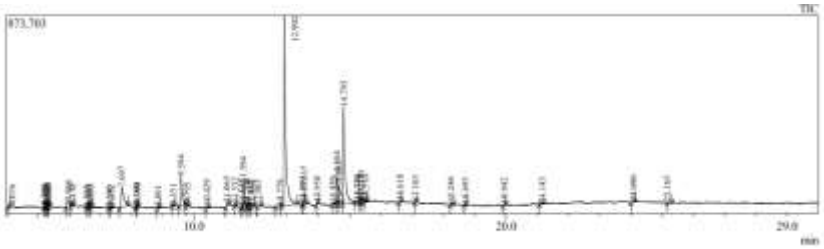

Figure 3: Peaks for Spot 3 of the ethanol extract

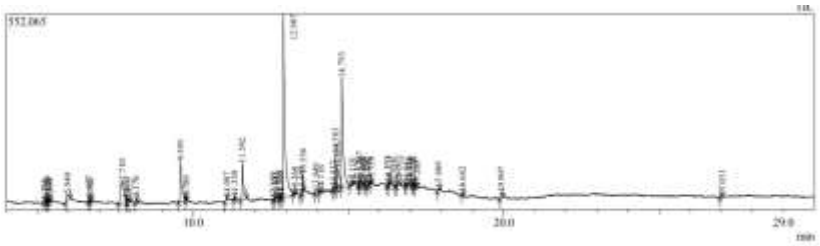

Figure 4: Peaks for Spot 4 of the ethanol extract

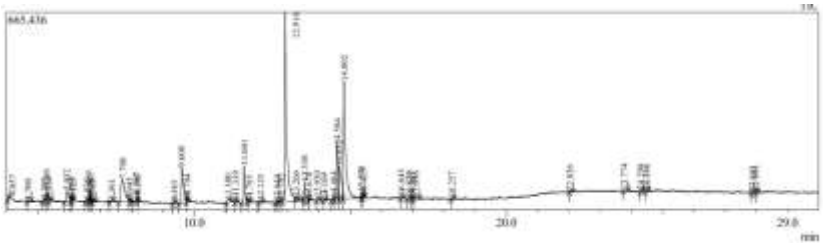

Figure 5: Peaks for Spot 5 of the ethanol extract

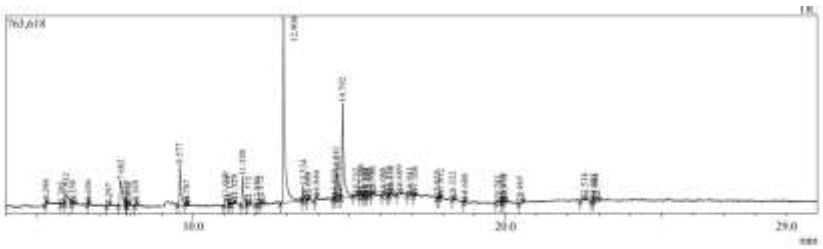

Figure 6: Peaks for Spot 6 of the ethanol extract

Table 2 lists the tentative GC-MS library annotations obtained for the recovered ethanol-extract bands. These are library-based spectral matches and are not confirmed structural identifications (see Section 3.8.10 and 3.8.11); match-score percentages and retention indices were not recorded and are consequently not reported.

Table 2: Putatively identified compounds from the ethanol extract (GC-MS library annotation)

Spot no.	Tentative annotation	Molecular formula	Molecular weight	Peak area (%)	Retention time
1	Methyl palmitate (putative)	$C_{17}H_{34}O_2$	270.45 g/mol	0.65%	16.591 min
2	Cannflavin-related flavonoid (putative)	$C_{18}H_{16}O_5$	312.32 g/mol	0.47%	21.171 min
3	Oleanolic acid (putative)	$C_{30}H_{48}O_3$	456.70 g/mol	0.23%	24.090 min
4	Pyrrolidide-type compound (putative)	$C_{32}H_{61}NO$	475.84 g/mol	0.30%	17.108 min
5	Fatty-acid methyl ester (putative)	$C_{17}H_{34}O_2$	270.45 g/mol	0.42%	16.643 min
6	Fatty-acid methyl ester (putative)	$C_{17}H_{34}O_2$	270.45 g/mol	0.42%	16.643 min

4.4 Putatively Annotated Compounds — Hexane Extract

Table 3: Retardation factor for the separation achieved using hexane (solvent front = 8.2 cm)

Spot number	Distance moved by compound (cm)	Retardation factor (Rf)
1	5.0	0.6
2	3.7	0.5
3	3.9	0.5
4	1.6	0.2
5	1.1	0.1

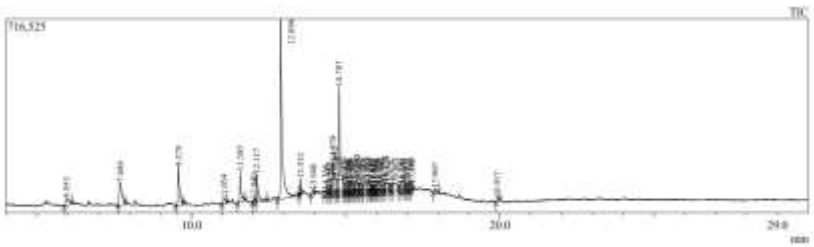

Figure 7: Peaks for Spot 1 of the hexane extract

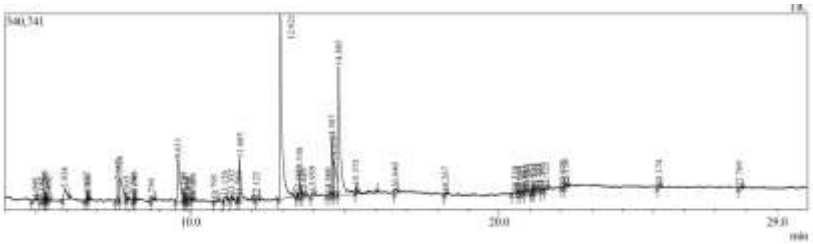

Figure 8: Peaks for Spot 2 of the hexane extract

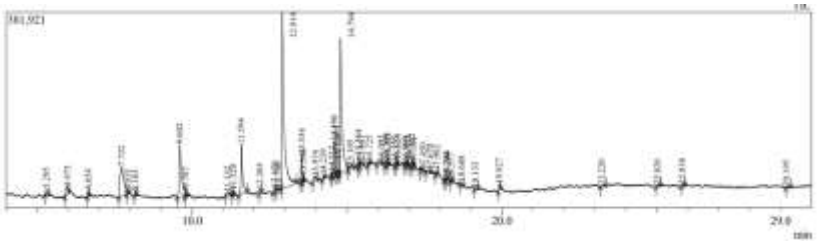

Figure 9: Peaks for Spot 3 of the hexane extract

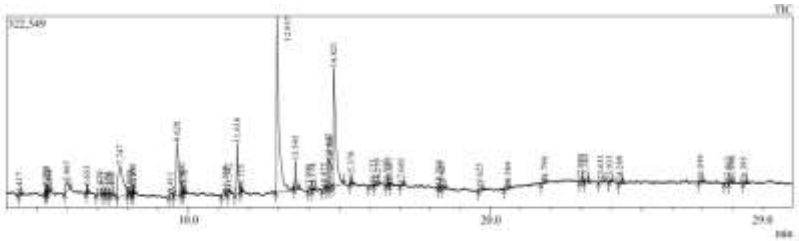

Figure 10: Peaks for Spot 4 of the hexane extract

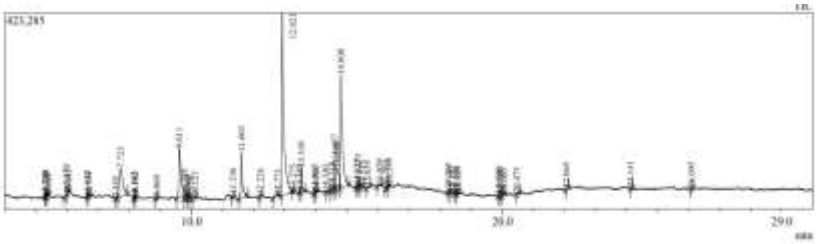

Figure 11: Peaks for Spot 5 of the hexane extract

Table 4: Putatively identified compounds from the hexane extract (GC-MS library annotation)

Spot no.	Tentative annotation	Molecular formula	Molecular weight	Peak area (%)	Retention time
1	Eicosane (putative alkane)	$C_{20}H_{42}$	282.55 g/mol	2.12%	16.325 min
2	Methyl palmitate (putative)	$C_{17}H_{34}O_2$	270.45 g/mol	26.07%	12.921 min
3	Uncertain — library suggestion: N,N-5α-cholestan-6β-amine (see §4.7)	$C_{27}H_{49}N$	387.69 g/mol	0.36%	18.377 min
4	Methyl palmitate (putative)	$C_{17}H_{34}O_2$	270.45 g/mol	27.92%	12.937 min
5	Uncertain — library suggestion: gorgost-5-en-3-ol, silylated derivative (see §4.7)	$C_{30}H_{52}OSi$	456.82 g/mol	0.34%	≈ 4.26 min

4.5 Putatively Annotated Compounds — Ethyl Acetate Extract

Table 5: Retardation factor for the separation achieved using ethyl acetate (solvent front = 8.0 cm)

Spot number	Distance moved by compound (cm)	Retardation factor (Rf)
1	5.3	0.7
2	4.2	0.5
3	3.0	0.4
4	1.3	0.2

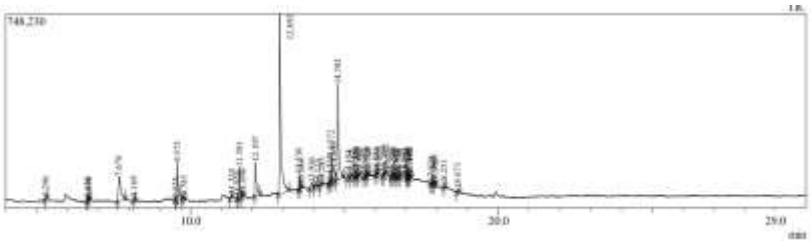

Figure 12: Peaks for Spot 1 of the ethyl acetate extract

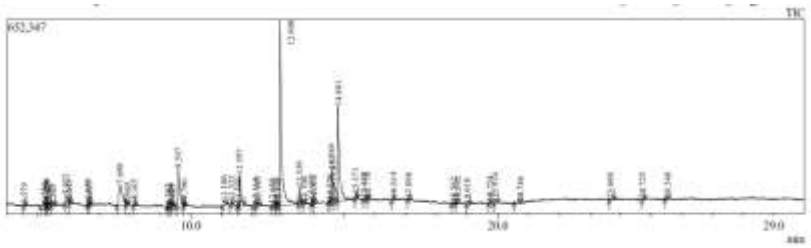

Figure 13: Peaks for Spot 2 of the ethyl acetate extract

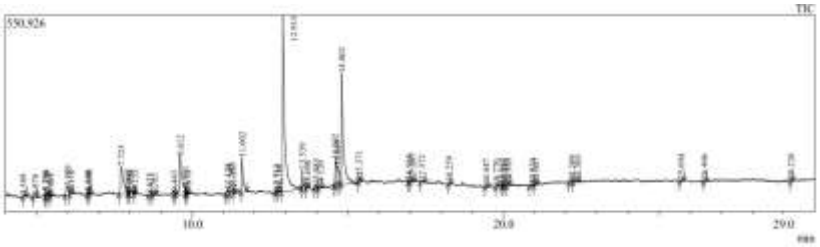

Figure 14: Peaks for Spot 3 of the ethyl acetate extract

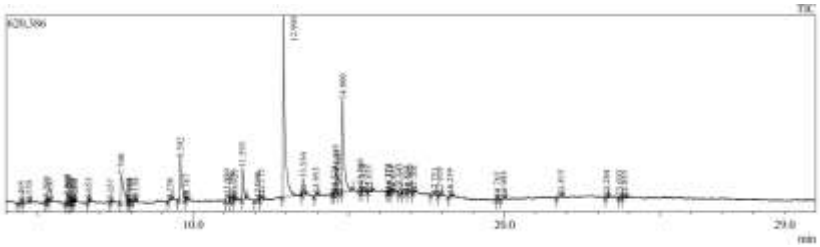

Figure 15: Peaks for Spot 4 of the ethyl acetate extract

Table 6: Putatively identified compounds from the ethyl acetate extract (GC-MS library annotation)

Spot no.	Tentative annotation	Molecular formula	Molecular weight	Peak area (%)	Retention time
1	Heneicosane (putative alkane)	$C_{21}H_{44}$	296.57 g/mol	0.48%	17.095 min
2	Methyl palmitate (putative)	$C_{17}H_{34}O_2$	270.45 g/mol	26.07%	12.921 min
3	Uncertain — library suggestion: 10-gingerol, disilylated derivative (see §4.7)	$C_{26}H_{48}O_4Si_2$	480.84 g/mol	0.29%	22.209 min
4	Methyl palmitate (putative)	$C_{17}H_{34}O_2$	270.45 g/mol	32.57%	12.905 min

4.6 Comparative Chromatographic Performance of the Three Extracts

The choice of extraction solvent influenced both the number of TLC bands recovered and the pattern of GC-MS annotations obtained. Under the hexane:ethyl acetate (7:3) TLC mobile-phase system used throughout this study, the ethyl acetate extract produced the cleanest chromatographic profile, with four clearly separated spots and no co-elution. It should be noted that ethyl acetate, ethanol and hexane were used here as extraction solvents, while the TLC separation itself was carried out using a fixed hexane:ethyl acetate (7:3) mobile phase; the cleaner separation is therefore a property of how the ethyl acetate extract behaved under that mobile-phase system, rather than evidence that ethyl acetate is inherently a "better TLC solvent" in a general sense.

By contrast, the ethanol extract was the only extract in which bands tentatively annotated as a Cannflavin-related flavonoid and as oleanolic acid were detected, despite showing co-elution

between two bands at Rf 0.4. The hexane and ethyl acetate extracts were associated mainly with putative alkanes, fatty-acid esters and sterol-related compounds.

4.7 Putatively Annotated Compounds and Their Reported Antibacterial Relevance

Compound tentatively annotated as a Cannflavin-related flavonoid (Spot 2, Rf 0.5, ethanol extract)

The GC-MS spectral library suggested a best match consistent with a Cannflavin-type flavonoid (structurally similar to 2',4'-dimethoxy-3-hydroxy-6-methylflavone). Flavonoids of this general type are not widely reported outside Cannabis in the literature reviewed for this study, so the compound is described here as tentatively annotated as a "Cannflavin-related flavonoid" rather than confirmed as unique to Cannabis sativa or definitively identified as Cannflavin itself; this description should not be used without qualification unless supported by a reliable literature source specific to this exact compound, library match percentage, molecular-ion data and comparison with published mass spectra, none of which were recorded in this study. This band was associated with the largest GC-MS peak area (0.47%) among the ethanol-extract bands, but peak area reflects relative abundance in the GC-MS run and is not a measure of antibacterial potency; no MIC, MBC or purified-compound testing was performed to establish how potent this constituent actually is relative to the others. It is therefore described here as a candidate antibacterial constituent associated with an active TLC band, rather than as "the most potent antibacterial compound" in the extract. Possible mechanisms reported in the literature for structurally related flavonoids include inhibition of bacterial enzymes such as DNA gyrase and topoisomerase IV, disruption of bacterial cell walls, and chelation of metal ions essential for bacterial growth; these mechanisms have not been directly demonstrated for the specific compound recovered in this study and are cited here as plausible mechanisms reported for related compounds in other species, not as mechanisms confirmed in this project.

Compound tentatively annotated as oleanolic acid (Spot 3, Rf 0.4, ethanol extract)

The library match for this band was consistent with oleanolic acid, a pentacyclic triterpenoid with antibacterial activity reported in the wider literature, including activity against Gram-positive and Gram-negative bacteria and some MRSA strains in other studies. As with the flavonoid band

above, this annotation is a tentative library match rather than a confirmed identification, and the antibacterial mechanisms often attributed to oleanolic acid in the literature — membrane disruption, enzyme inhibition and anti-inflammatory activity — should be understood as mechanisms reported for oleanolic acid generally, not as mechanisms confirmed for the specific band recovered in this study.

Methyl palmitate and other fatty-acid esters

Fatty-acid methyl esters, including methyl palmitate and putative methyl stearate/methyl oleate-type compounds, were tentatively annotated in multiple bands across all three extracts. These compounds are generally reported to show, at most, moderate antibacterial activity, plausibly through membrane disruption and hydrophobic interaction with bacterial membranes; given their comparatively large peak areas in several bands (for example, 26–33% in some hexane and ethyl acetate bands), their presence may also reflect general lipid content of the leaf extract rather than the primary source of antibacterial activity.

4.8 Co-elution as an Analytical Limitation

Two bands in the ethanol extract co-eluted at Rf 0.4, and two bands in the hexane extract co-eluted at Rf 0.5. Where bands co-elute, attribution of any observed antibacterial activity to a single constituent becomes uncertain, because more than one compound may be contributing to (or masking) the activity observed at that position. This is treated as a genuine analytical limitation rather than a minor technical detail. Recommended follow-up approaches include adjusting the mobile-phase solvent ratio, testing an alternative solvent system, applying two-dimensional TLC, or proceeding to preparative TLC or column chromatography to fully resolve co-eluting bands before further compound attribution is attempted.

4.9 Assignments Requiring Additional Verification

Several GC-MS library suggestions obtained from the hexane and ethyl acetate extracts are chemically unusual and are flagged here for caution rather than presented as established findings. These include a suggestion of N,N-5α-cholestan-6β-amine, a silicon-containing formula suggested for a gorgost-5-en-3-ol-type compound, and a disilylated formula suggested for a 10-gingerol-type compound. Silicon-containing formulae in particular are consistent with trimethylsilyl

derivatization products, column-bleed artefacts, or simply weak/low-confidence library matches rather than genuine plant constituents. Because match scores, retention indices and derivatization procedures were not recorded (Section 3.8.10), this report cannot distinguish with confidence between a genuine trace constituent, a derivatization artefact, contamination, or a column-bleed-related assignment for these three bands. These three annotations are therefore explicitly excluded from the antibacterial-relevance discussion in Section 4.7 and should be treated as unverified until re-analysed with full method documentation and, ideally, a higher-confidence match-score threshold.

4.10 Summary Linkage: Organism, Active Band and GC-MS Annotation

The table below summarizes, to the extent the available data allow, the linkage between test organism, active Rf band and GC-MS annotation. Several cells are marked "not differentiated" because the bioautography assay in this study recorded overall inhibition patterns per extract rather than organism-specific zone measurements for each individual band; this is itself one of the key limitations identified below.

Extract	Test organism(s)	Active Rf (approx.)	Zone size/intensity	GC-MS annotation
Ethanol	S. aureus, E. coli (not differentiated)	0.5	Not quantified	Cannflavin-related flavonoid (putative)
Ethanol	S. aureus, E. coli (not differentiated)	0.4	Not quantified	Oleanolic acid (putative)
Hexane	S. aureus, E. coli (not differentiated)	Not differentiated between co-eluting bands	Not quantified	Methyl palmitate / uncertain sterol-type (putative)
Ethyl acetate	S. aureus, E. coli (not differentiated)	0.5, 0.4	Not quantified	Methyl palmitate / 10-gingerol-type (uncertain)

4.11 Limitations of the Study

The following limitations should be considered when interpreting the results presented in this chapter:

i. Compound identification relied entirely on GC-MS spectral-library matching; no authentic standards, retention-index confirmation or complementary spectroscopic techniques (e.g., NMR, LC-MS/MS) were used, so all compound names should be read as tentative annotations rather than confirmed identifications.

ii. No MIC, MBC, inhibition-zone measurements, or dose-response data were generated for individual isolated compounds; consequently, no compound can be described as "most potent" on the basis of the data collected.

iii. Positive, negative, growth and sterility controls were not systematically documented for every bioautography run, limiting the confidence with which inhibition can be attributed solely to plant-derived compounds.

iv. The agar diffusion assay specified in Objective (ii) was not completed to reportable standard; the results in this chapter derive from TLC-bioautography only.

v. Bacterial isolates were not characterized for strain identity or antibiotic-resistance profile, so claims cannot be made about activity against confirmed multidrug-resistant (e.g., MRSA, ESBL-producing) strains.

vi. Extraction yield was not determined, so solvent comparisons are limited to chromatographic and GC-MS profile rather than quantity of material extracted.

vii. Each extract and bioautography assay was analysed once rather than in replicate, so no measure of variability or reproducibility is available.

viii. GC-MS instrument parameters and identification criteria (match-score cut-off, retention index, library used) were not fully documented, limiting reproducibility of the GC-MS results.

ix. Co-elution of bands in the ethanol and hexane extracts means that activity attributed to a given Rf position may reflect more than one co-migrating compound.

4.12 Implications for Future Antibacterial Drug Discovery

Notwithstanding the limitations above, the tentative annotation of a Cannflavin-related flavonoid and of oleanolic acid in the ethanol extract is a useful exploratory finding. If confirmed by purification and structural analysis, a Cannflavin-type scaffold would be of particular interest because comparable flavonoids are not commonly reported outside Cannabis, and literature on structurally related flavonoids suggests multiple possible mechanisms of action that could, in principle, make resistance development more difficult. Oleanolic acid, while previously reported in other plant species, would represent a further confirmed contributor to the antibacterial profile of Cannabis sativa if the annotation is verified. These findings should be regarded as a starting point for confirmatory work rather than as an established basis for drug-development claims.

CHAPTER FIVE: CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

This study demonstrated that ethanol, ethyl acetate and hexane extracts of Cannabis sativa leaves contain chromatographically separable constituents associated with antibacterial activity against Staphylococcus aureus and Escherichia coli. TLC-bioautography localized active bands, and GC-MS analysis of the recovered fractions generated tentative library-based annotations, including a compound provisionally annotated as a Cannflavin-related flavonoid, a compound provisionally annotated as oleanolic acid, several fatty-acid esters, putative alkanes, and a small number of sterol-related annotations that require further verification (Section 4.9).

The ethyl acetate extract produced the cleanest TLC profile under the chromatographic conditions used in this study, while the ethanol extract yielded active fractions containing the constituents of greatest apparent interest, namely the putative Cannflavin-related flavonoid and putative oleanolic acid, despite co-elution between two bands at Rf 0.4. Because compound identification in this study relied on GC-MS spectral-library matching alone, and because the antibacterial potency of the recovered constituents was not quantified through MIC, MBC or dose-response testing, the findings of this study should be regarded as preliminary and exploratory rather than confirmatory. They nonetheless provide useful, scientifically grounded support for the traditional use of Cannabis sativa in managing bacterial infections and identify specific, named candidate bands — most notably the putative Cannflavin-related flavonoid — that merit dedicated purification, structural confirmation, and quantitative antibacterial testing in future work before any claims of drug-development potential can be made.

5.2 Recommendations

The recommendations below are ordered to prioritize validation of the present findings ahead of translational or drug-development claims.

i. Optimize the TLC separation system, particularly to resolve the co-eluting bands observed in the ethanol extract (Rf 0.4) and hexane extract (Rf 0.5), for example by adjusting the mobile-phase ratio, trialling an alternative solvent system, or applying two-dimensional TLC.

ii. Repeat the bioautography assay in replicate, with positive controls (a standard antibiotic), negative controls (extraction solvent), growth controls (inoculated plate without extract) and sterility controls (uninoculated plate) included in every run, so that inhibition can be attributed to plant constituents with greater confidence.

iii. Measure inhibition-zone diameters, or another suitable quantitative bioautography endpoint, for each active band against each test organism, rather than relying on visual/qualitative comparison alone.

iv. Purify the main active bands (particularly the putative Cannflavin-related flavonoid and putative oleanolic acid) using column chromatography and/or preparative TLC to obtain pure compounds for confirmatory testing.

v. Confirm the structures of purified compounds using higher-confidence techniques such as NMR spectroscopy and/or LC-MS/MS, together with comparison against authentic reference standards where available, and report GC-MS match scores and retention indices for any further GC-MS work.

vi. Determine the MIC and MBC of each purified compound against Staphylococcus aureus and Escherichia coli, and, where feasible, against characterized multidrug-resistant strains (e.g., confirmed MRSA or ESBL-producing E. coli isolates).

vii. Compare the antibacterial activity of purified compounds against standard reference antibiotics under the same assay conditions.

viii. Complete the agar diffusion assay described in the original study objectives, or formally revise the stated objectives to match the screening methods actually completed, so that the dissertation's objectives and reported results are fully aligned.

ix. Determine extraction yield for each solvent (mass of dry extract relative to mass of dry plant powder) to allow quantitative comparison of solvent performance.

x. Evaluate the cytotoxicity and selectivity of purified compounds before considering any translational application.

xi. For compounds poorly suited to GC-MS (e.g., larger, thermally labile or highly polar constituents), consider LC-MS/MS or NMR-based approaches as a complement to GC-MS.

xii. Resazurin dye is recommended as a rapid and sensitive indicator for detecting antibacterial activity in TLC-bioautography assays, since living bacteria reduce blue resazurin to pink resorufin while antibacterial compounds prevent this reduction, producing clear inhibition zones; this element of the original methodology performed well and should be retained in follow-up studies.

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APPENDICES



Figure 16: Grinding of Cannabis sativa leaves



Figure 17: Measuring of the different extraction solvents



Figure 18: TLC-bioautography and UV observations

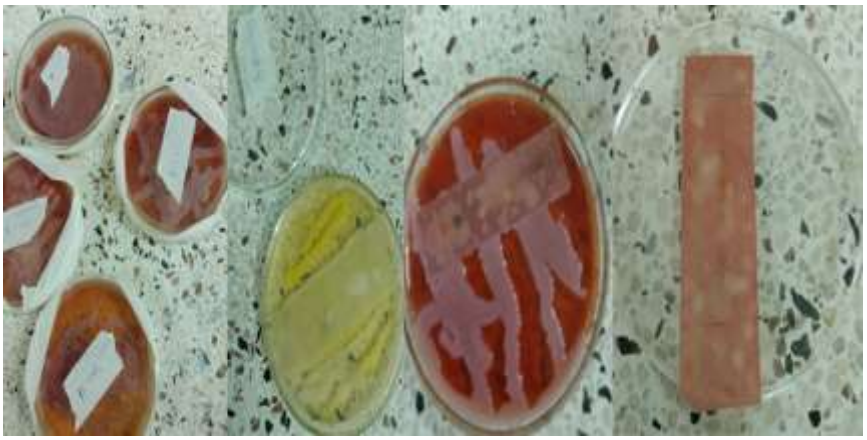

Figure 19: Bacterial culturing

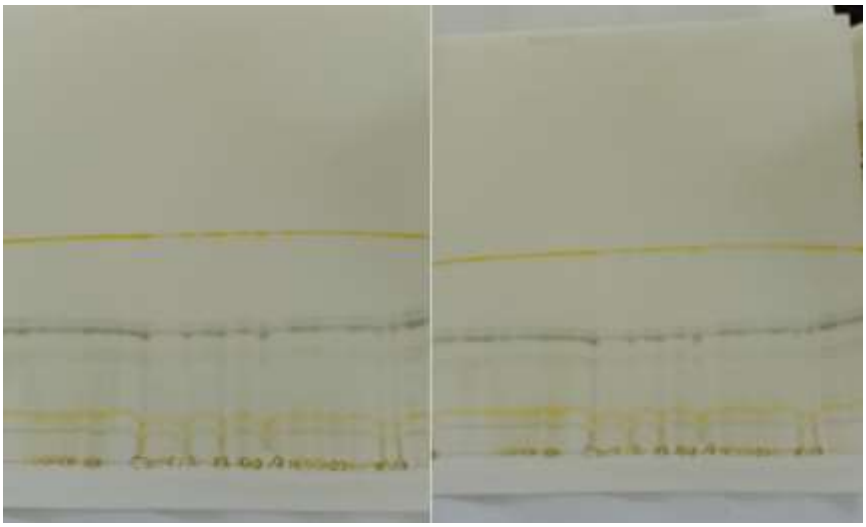

Figure 20: TLC plates with spots scraped for GC-MS analysis