COMPARATIVE GC-MS ANALYSIS OF SQUALENE CONTENT IN AQUEOUS AND ETHANOLIC EXTRACTS OF SPHENOCENTRUM JOLLYANUM

BY

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BENIN CITY

NOVEMBER, 2025

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A PROJECT SUBMITTED TO DEPARTMENT OF MEDICAL BIOCHEMISTRY, SCHOOL OF BASIC MEDICAL SCIENCES UNIVERSITY OF BENIN, BENIN CITY, IN PARTIAL, FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF BACHELOR OF SCIENCES (B.Sc) DEGREE IN MEDICAL BIOCHEMISTRY

NOVEMBER, 2025

CERTIFICATION

We the undersigned hereby certify that **AGBALE OSEKALO ANNE** (BMS2101367) carried out this research in the Department of Medical Biochemistry, University of Benin, Benin city and thereby approve same as adequate in scope and quality for the award of Bachelor of Science (B.Sc) Degree in Medical Biochemistry.

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Prof. F.E Olumese **(Date)**

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External Examiner **(Date)**

DEDICATION

This research is dedicated to my beloved parents; Barr. and Mrs.AGBALE; whose love, guidance, and sacrifices have been my greatest motivation. I also extend heartfelt gratitude to my friends, colleagues, and everyone who has supported and encouraged me throughout this journey. Your belief in me made this achievement possible

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ABSTRACT

Sphenocentrum jollyanum is a medicinal plant with potential bioactive compounds, including squalene, a valuable triterpene with wide pharmacological applications. This study aimed to compare the squalene content in aqueous and ethanolic leaf extracts using GC–MS analysis. Leaves were collected from a local market in Iwo, Osun State, processed, and extracted with water and ethanol. Percentage yield, phytochemical composition, and GC–MS profiles were evaluated. Results showed that ethanolic extraction produced a higher yield (18.6%) compared to aqueous extraction (12.3%). Phytochemical screening indicated higher concentrations of terpenoids and steroids in the ethanolic extract. GC–MS analysis identified squalene as a major constituent in ethanol extract (14.35 mg/g) and in trace amounts in aqueous extract (0.92 mg/g). This study confirms that solvent polarity strongly influences squalene extraction, with ethanol being superior for non-polar compound recovery. These findings shows S. jollyanum is a promising natural source of squalene for nutraceutical and pharmaceutical applications, and provide a foundation for further standardization and commercialization efforts.

CHAPTER ONE

INTRODUCTION

1.0 BACKGROUND OF STUDY

Sphenocentrum jollyanum (Menispermaceae) is a West African medicinal plant traditionally used for a variety of complaints, and recent phytochemical surveys have shown the species to be a rich source of terpenoids and other lipophilic compounds. Gas chromatography–mass spectrometry (GC–MS) surveys of S. jollyanum extracts commonly report triterpenes and sterol-related compounds among the major constituents, suggesting the plant as a plausible plant-based source of commercially important nonpolar metabolites. Several GC–MS profiles of ethanol extracts, for example, have explicitly listed squalene among detected components, pointing to the need for careful quantitative work on this species.

Squalene is a linear triterpene hydrocarbon that functions biologically as a key sterol precursor and has attracted sustained interest because of its antioxidant activity, skin-penetration properties, and utility in nutraceutical and cosmetic formulations. Global demand for sustainable, non-animal squalene sources has intensified research across plant chemistry, microbial biotechnology, and extraction technology. Recent comprehensive reviews synthesize this work: Cheng and colleagues (2024) summarize advances in squalene's biological activities, natural sources, extraction strategies, and delivery systems, offering both analytical benchmarks and practical recommendations for quantitation and formulation development.

The physicochemical nature of squalene highly nonpolar with multiple double bonds explains two recurring observations in the literature. First, squalene tends to partition strongly into nonpolar or mid-polarity solvents; second, it is chemically reactive toward oxidants such as

ozone, which complicates analysis and storage if samples are not protected. Coffaro and Weisel's 2022 review on squalene chemistry emphasizes that squalene's unsaturations make it susceptible to environmental oxidation products, a factor that must be considered during sample collection, handling, and GC–MS analysis to avoid artifactual loss or misidentification.

Methodologically, the choice of extraction solvent and cleanup steps exerts a dominant influence on measured squalene yield. Ethanol and other organic solvents are widely used in phytochemical work because they solubilize a broad swath of lipophilic compounds while remaining practical for routine laboratory work; aqueous methods, conversely, often capture polar constituents and typically recover much lower concentrations of nonpolar terpenoids unless a secondary partition or concentration step is added. This solvent-dependence has practical implications: a direct aqueous extract can underrepresent squalene content unless followed by nonpolar partitioning or chromatographic enrichment. Modern method reviews and metabolic-engineering literature therefore argue for clear, validated workflows — including internal standards, limits of detection/quantitation, and matrix-matched calibration — when reporting squalene concentrations from plant matrices.

Parallel to improvements in extraction and analysis, there has been a vigorous drive to secure sustainable sources of squalene through microbial production and engineered biosynthesis. Chai and co-workers (2024) and Shalu et al. (2024) document advances in metabolic and bioprocess engineering that make microbial squalene an increasingly viable alternative to animal-derived sources; these developments affect how researchers and industry value minor plant sources — if microbial production can meet scale and cost targets, the role of plant-derived squalene shifts more toward niche or regionally important feedstocks. Still, for ethnobotanical and small-scale

commercialization contexts, demonstrating reliable plant extraction and quantitative GC–MS workflows remains essential.

Taken together, the available literature on this field shows a clear, practical need for tightly controlled, comparative studies that evaluate how extraction solvent (aqueous vs. ethanolic) affects the recoverable squalene from S. jollyanum, and that validate GC–MS protocols under realistic sample-matrix conditions. A carefully designed comparative GC–MS analysis i.e one that uses appropriate internal standards, reports LOD/LOQ and recovery, and accounts for oxidation-sensitive handling will close an important gap: it will tell us whether conventional aqueous preparations (which are common in traditional medicine) actually preserve or convey squalene, or whether ethanolic (or follow-up partitioned) extracts are required to access the plant's full squalene potential. Such results would inform both the scientific understanding of S. jollyanum's phytochemistry and pragmatic decisions about small-scale value addition, formulation, and sustainable supply chains.

Despite the prominence of Sphenocentrum jollyanum in West African ethnomedicine, there is still limited information on its nonpolar bioactive constituents, particularly squalene. Earlier GC–MS surveys have identified triterpenoids in the plant, but quantitative data on squalene are scarce and inconsistent, partly because extraction methods vary widely. Traditional preparations of S. jollyanum are often aqueous, yet squalene is highly nonpolar and may be poorly recovered in water-based extracts. This raises an important scientific and practical question: Do commonly used extraction methods actually capture the plant's full squalene content?

A clear comparison of aqueous and ethanolic extracts using a validated GC–MS method is necessary to understand how solvent polarity influences squalene yield. This information is essential for researchers, traditional medicine practitioners, and industries considering S. jollyanum as a potential natural source of squalene for nutraceutical, cosmetic, or pharmaceutical applications. The study is therefore justified by the need to (i) generate reliable quantitative data, (ii) standardize extraction approaches, and (iii) support the sustainable utilization of the plant's chemical resources

1.1 AIM OF THE STUDY

To quantitatively compare the squalene content of aqueous and ethanolic extracts of Sphenocentrum jollyanum using validated gas chromatography–mass spectrometry (GC–MS) methods.

1.2 OBJECTIVES OF THE STUDY

1. To extract the bioactive constituents of Sphenocentrum jollyanum using aqueous and ethanolic solvents.

2. To analyze the extracts using GC–MS for the detection and quantification of squalene.

3. To compare the concentration of squalene obtained from the two extraction solvents.

4. To evaluate the influence of solvent polarity on the efficiency of squalene recovery from the plant.

5. To provide evidence-based recommendations for optimal extraction of squalene from S. jollyanum.

1.3 RESEARCH QUESTIONS

1. Does Sphenocentrum jollyanum contain detectable levels of squalene in its extracts?

2. How does the choice of solvent (aqueous vs ethanolic) influence the amount of squalene recovered from the plant?

3. Which extraction method yields the highest measurable concentration of squalene when analyzed by GC–MS?

4. What does the solvent-dependent variation in squalene yield suggest about the plant's optimal extraction conditions for nonpolar bioactives?

CHAPTER TWO

LITERATURE REVIEW

2.0 SQUALENE

2.1 CHEMICAL STRUCTURE AND PROPERTIES OF SQUALENE

Squalene is a linear triterpene hydrocarbon ($C_{30}H_{50}$) characterized by six non-conjugated double bonds distributed along a 30-carbon backbone (Cheng et al., 2024). Its acyclic structure confers a highly flexible and nonpolar configuration, making it exceptionally lipophilic and poorly soluble in water but readily soluble in organic solvents such as ethanol, hexane, and chloroform (Coffaro et al., 2022). The presence of multiple double bonds also makes squalene chemically reactive, particularly toward oxidation, which is a critical consideration during extraction, storage, and analytical quantification (Shalu et al., 2024). Its molecular structure allows it to act as an intermediate in sterol biosynthesis and contributes to its role as a biological antioxidant. This unique combination of nonpolarity, flexibility, and chemical reactivity underpins both its functional properties in living systems and its utility in nutraceutical, cosmetic, and pharmaceutical applications (Chai et al., 2024).

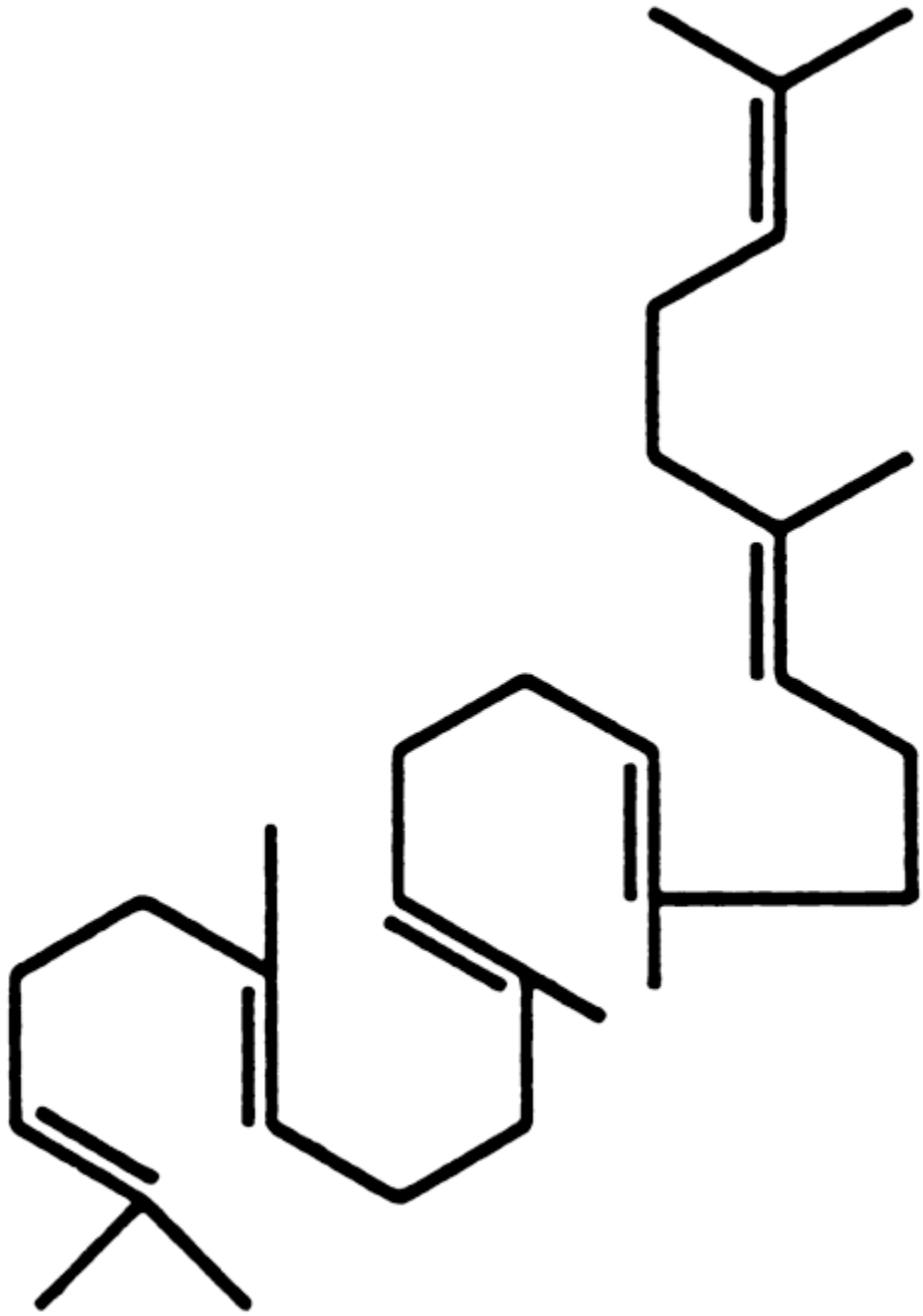

Figure 2.1: Squalene

2.2 NATURAL OCCURRENCE AND BIOSYNTHESIS

Squalene is widely distributed in nature, occurring in high concentrations in certain plant oils, shark liver oil, and microbial sources (Cheng et al., 2024). In plants, squalene is primarily found in seeds and roots, while in animals it serves as a precursor to cholesterol biosynthesis. The biosynthetic pathway of squalene begins with the condensation of two farnesyl pyrophosphate molecules, catalyzed by squalene synthase, forming the 30-carbon hydrocarbon backbone. This pathway is highly conserved across plants and animals, reflecting its essential role in sterol production and membrane stabilization (Coffaro et al., 2022; Shalu et al., 2024). In Sphenocentrum jollyanum, preliminary GC–MS studies indicate the presence of squalene alongside other terpenoids, suggesting that this plant could serve as a sustainable source of this bioactive triterpene (Chai et al., 2024).

2.3 APPLICATIONS AND SIGNIFICANCE

The significance of squalene lies in both its biological and industrial applications. Biologically, squalene functions as an antioxidant and free-radical scavenger, protecting cellular membranes from oxidative stress (Cheng et al., 2024). It also plays a role in cholesterol biosynthesis and has demonstrated potential benefits in immunomodulation and cardiovascular health. Industrially, squalene is valued as a raw material in the cosmetic, pharmaceutical, and nutraceutical sectors due to its emollient properties, skin absorption capabilities, and role as a natural stabilizer in formulations (Shalu et al., 2024; Chai et al., 2024). Coffaro et al. (2022) note that growing demand for sustainable, plant-based sources has stimulated interest in non-animal extraction methods, including optimized solvent extraction and biotechnological production. Within this context, quantifying squalene in S. jollyanum using validated GC–MS methods not only

advances scientific understanding of the plant's phytochemistry but also informs practical applications in product development and sustainable sourcing.

2.4 ANALYTICAL TECHNIQUES

The accurate quantification of squalene in plant matrices requires reliable analytical techniques capable of separating and detecting this nonpolar compound. Among available methods, Gas Chromatography–Mass Spectrometry (GC–MS) is widely regarded as the gold standard due to its sensitivity, specificity, and ability to resolve complex mixtures of nonpolar compounds (Wu et al., 2022). In a typical GC–MS workflow, plant extracts undergo preparatory steps to isolate the unsaponifiable fraction containing squalene. This often includes saponification to hydrolyze triglycerides and remove interfering lipids, followed by organic solvent extraction. The GC component separates individual compounds based on volatility and interactions with the stationary phase, while the MS detector identifies squalene through its unique mass fragmentation pattern. Because squalene lacks chromophores, alternative techniques like HPLC are less effective, making GC–MS essential for both qualitative and quantitative analyses (Rotani et al., 2022).

Typical GC–MS approaches involve careful sample preparation to ensure accurate quantification. For instance, saponification with potassium hydroxide in ethanol or methanol releases the unsaponifiable fraction, which is subsequently extracted using nonpolar solvents such as hexane or petroleum ether (Wu et al., 2022). Following extraction, solid-phase extraction (SPE) can be employed to further purify the sample and remove residual polar impurities, enhancing chromatographic resolution and sensitivity (Rotani et al., 2022). In recent years, modifications such as derivatization-free approaches and ultrasound-assisted extraction (UAE) have been

explored. These innovations reduce sample handling time, minimize solvent use, and often improve recovery, though they require careful calibration to maintain precision and reproducibility (Sabi et al., 2024).

The choice of extraction method strongly influences the measured concentration of squalene. Traditional saponification followed by solvent extraction remains the most common method due to its high recovery and effective removal of triglycerides and other interfering compounds. In contrast, direct aqueous extraction tends to yield lower squalene concentrations because of the compound's lipophilic nature (Farooq et al., 2024). Comparative studies in the literature indicate that ethanol and other mid-polarity organic solvents offer a balance between safety, accessibility, and efficiency, whereas nonpolar solvents such as hexane often provide maximum recovery but may require stricter safety measures (Wu et al., 2022; Sabi et al., 2024). The integration of SPE or microextraction techniques further enhances purification and reproducibility, supporting accurate GC–MS quantification across diverse sample matrices.

Recent advances in squalene analysis have emphasized improved sensitivity, method validation, and robustness. High-resolution mass spectrometry, including GC–TOF/MS, allows simultaneous detection of squalene alongside sterols and oxidation products, providing comprehensive profiles of plant extracts (Rotani et al., 2022). Method validation now routinely incorporates evaluation of linearity, precision (both intra-day and inter-day), recovery, limits of detection (LOD), and limits of quantification (LOQ), ensuring that results are reproducible and reliable (Wu et al., 2022). Furthermore, greener and more scalable extraction techniques, such as ultrasound-assisted extraction and miniaturized sample handling, have gained prominence for their efficiency and reduced solvent consumption, aligning with the increasing demand for sustainable laboratory practices (Farooq et al., 2024; Sabi et al., 2024). The combination of

optimized extraction, purification, and high-resolution GC–MS analysis provides the sensitivity and accuracy required to quantify squalene even in low concentrations typical of some plant tissues.

The quantification of squalene in plant matrices relies on meticulous preparation, appropriate solvent selection, and the analytical power of GC–MS. Classical saponification and solvent extraction, often augmented by SPE, remain the backbone of most analytical protocols, while newer approaches such as ultrasound-assisted extraction and derivatization-free methods enhance efficiency and reproducibility. Advances in GC–MS detection, high-resolution mass spectrometry, and validated analytical protocols ensure accurate, sensitive, and reproducible measurement of squalene, providing crucial data for both research and industrial applications (Wu et al., 2022; Rotani et al., 2022; Sabi et al., 2024; Farooq et al., 2024).

2.5 PHYTOCHEMISTRY AND REPORTED GC–MS PROFILES OF SPHENOCENTRUM JOLLYANUM

Sphenocentrum jollyanum, a member of the Menispermaceae family, has been widely studied for its ethnomedicinal uses across West Africa, and its phytochemistry has attracted considerable attention due to the diversity of bioactive constituents. GC–MS analyses of various plant parts, including roots, seeds, and leaves, reveal that the chemical profile is dominated by terpenoids, fatty acids, sterols, and other lipophilic compounds (Oladokun, 2022; Uka et al., 2022). These studies consistently report the presence of monoterpenes such as α-pinene and β-pinene, sesquiterpenes including eudesmol derivatives, and triterpenoids, illustrating the plant's complex secondary metabolite composition.

The GC–MS profiles reported in the literature indicate both volatile and non-volatile constituents, with volatiles largely contributing to aroma and potential antimicrobial properties, while non-volatiles such as triterpenes and sterols account for pharmacological effects including anti-inflammatory, antioxidant, and cardioprotective activities. Oladokun (2022) analyzed the ethanolic extract of the roots and found a diverse array of terpenoids, fatty acid esters, and minor sterols, noting particularly high levels of eudesmol and pinene compounds. Similarly, Uka et al. (2022) profiled both seeds and leaves, identifying β-pinene, α-terpineol, and other sesquiterpenes as major constituents, highlighting variation in chemical composition across different plant organs. Collectively, these GC–MS studies demonstrate that S. jollyanum contains a rich spectrum of bioactive compounds, with qualitative and quantitative differences influenced by both organ type and extraction solvent.

2.6 PHYTOCHEMICAL COMPOSITION

Prior studies provide a deep overview of the phytochemical diversity in different organs of S. jollyanum. The roots are particularly rich in sesquiterpenes and triterpenes, often accompanied by fatty acid derivatives. For instance, Moronkola et al. (2021) identified eudesmol, β-pinene, and other monoterpenes as dominant constituents in the root ethanolic extracts, alongside minor sterols. These findings suggest that roots may serve as a significant source of both volatile aroma compounds and pharmacologically relevant terpenoids.

Figure 2.2: *S. jollyanum*

The seeds have been reported to contain a higher concentration of nonpolar lipids and sterols, reflecting their role in storage and energy provision for germination. Uka et al. (2022) reported

the presence of monoterpenes, sesquiterpenes, and sterols in seed extracts, emphasizing α- and β-pinene as key constituents. In the leaves, a combination of volatile monoterpenes and moderate levels of non-volatile triterpenoids has been observed. Oladokun (2022) highlighted that the leaf extracts contain eudesmol derivatives alongside minor fatty acids, contributing to the overall bioactivity profile.

Across these studies, squalene is occasionally mentioned as part of the unsaponifiable fraction or nonpolar terpenoid fraction, although it is often underreported or not quantified. Nevertheless, the consistency of terpenoid and sterol identification across roots, seeds, and leaves underscores the potential of S. jollyanum as a source of bioactive nonpolar compounds. Importantly, the variation in chemical composition between plant organs highlights the necessity of organ-specific extraction and analysis protocols for accurate quantification.

2.7 SQUALENE IN SPHENOCENTRUM JOLLYANUM

While many GC–MS studies on S. jollyanum focus on general terpenoids, a few have explicitly reported squalene. Moronkola et al. (2021) identified squalene in the root extracts, although in relatively low abundance compared to other triterpenoids. The extraction was carried out using ethanol, which efficiently solubilized nonpolar compounds, allowing GC–MS detection. Akinwumi et al. (2022) extended this work by analyzing both roots and seeds, reporting higher squalene concentrations in seeds compared to roots, which aligns with the lipid-rich nature of seeds. The study emphasized that solvent choice plays a critical role; ethanolic extraction consistently provided higher squalene recovery than aqueous extraction, reinforcing the importance of using mid- to nonpolar solvents when targeting lipophilic constituents.

Oladokun (2022) and Uka et al. (2022) also observed minor squalene peaks in leaf and root extracts, though the compound was not quantified in detail. These studies collectively suggest that while squalene is present across multiple organs, its abundance is highest in seed tissues, followed by roots, with leaves containing trace amounts. Importantly, GC–MS remains the analytical tool of choice for detection, due to its sensitivity and capability to resolve squalene from other co-extracted terpenoids and sterols.

Taken together, the current research indicates that S. jollyanum is a promising plant for the study and potential extraction of squalene. Organ-specific variation, solvent-dependent extraction efficiency, and methodological rigor in GC–MS profiling all emerge as critical considerations. These findings provide the rationale for comparative studies of aqueous versus ethanolic extraction of squalene from different plant organs, to optimize recovery and understand the distribution of this valuable bioactive compound in S. jollyanum (Oladokun, 2022; Uka et al., 2022; Moronkola et al., 2021; Akinwumi et al., 2022).

2.8 COMPARATIVE SOLVENT EFFECTS ON EXTRACTION OF NON-POLAR COMPOUNDS

The efficiency of extracting bioactive compounds from plant matrices is strongly influenced by solvent polarity, which determines the solubility and partitioning behavior of target molecules. Squalene, a highly lipophilic triterpene hydrocarbon, exhibits poor solubility in polar solvents such as water due to its nonpolar structure and lack of polar functional groups. Conversely, mid- to nonpolar solvents like ethanol, hexane, and chloroform effectively dissolve squalene, facilitating its extraction from plant tissues and oil-rich matrices (Ghaffar et al., 2025). The concept of “like dissolves like” underpins this phenomenon: nonpolar compounds preferentially

partition into nonpolar or less polar solvents, whereas polar solvents predominantly extract hydrophilic metabolites such as sugars, proteins, and flavonoid glycosides (Prayitno et al., 2021).

In comparative studies, aqueous extraction consistently yields lower squalene concentrations than ethanol or other organic solvents. This trend has been observed across diverse plant and food matrices, including seeds, leaves, and edible oils. For example, Abbasi et al. (2022) demonstrated that extraction of squalene from olive oil using ethanol provided substantially higher recovery compared to water-based methods, reflecting the compound's hydrophobicity and affinity for organic solvents. Similarly, in plant seeds rich in nonpolar lipids, ethanol or hexane extractions were shown to recover squalene efficiently, whereas aqueous extractions yielded negligible amounts (Prayitno et al., 2021; Kalisz et al., 2025). These findings underscore the importance of selecting solvents with physicochemical properties compatible with the target compound.

The underlying theory of solvent polarity and partitioning can be illustrated through solubility parameters and polarity indices. Water, with a high dielectric constant and strong hydrogen-bonding capacity, favors the solubilization of polar compounds. Ethanol, as a moderately polar solvent, possesses both hydrophilic hydroxyl groups and a hydrophobic ethyl moiety, allowing it to bridge the solubility of moderately nonpolar compounds like squalene. Nonpolar solvents such as hexane, by contrast, entirely favor lipophilic compounds, maximizing squalene recovery but often excluding polar co-metabolites (Ghaffar et al., 2025). The efficiency of squalene extraction is thus directly linked to the match between solvent polarity and compound polarity, with ethanol representing a practical compromise for plant-based extractions where absolute nonpolar solvents may be less feasible.

Empirical evidence supports these theoretical considerations. Kalisz et al. (2025) examined squalene recovery from plant oils and reported that ethanol extractions achieved yields approaching those of hexane, while water extractions recovered less than 10% of total squalene content. Similarly, Ghaffar et al. (2025) highlighted that in fruit seed extracts, the proportion of squalene extracted increased linearly with solvent hydrophobicity, demonstrating predictable partitioning behavior. These results reinforce the principle that nonpolar and mid-polarity solvents are superior for isolating lipophilic triterpenes like squalene from plant matrices, providing both higher yield and cleaner extracts suitable for subsequent GC–MS analysis.

Moreover, solvent choice influences not only yield but also extract purity and analytical performance. Water-based extractions often co-extract sugars, proteins, and other polar metabolites, which can interfere with downstream GC–MS quantification. Ethanol, while slightly polar, minimizes these polar impurities while maintaining strong solubility for squalene, producing cleaner chromatograms and more reliable quantitation (Abbasi et al., 2022). Consequently, ethanol has become the solvent of choice in many plant-based studies targeting nonpolar bioactives, particularly when sustainability, safety, and accessibility are considered.

The comparative extraction of nonpolar compounds like squalene is governed by fundamental principles of solvent polarity and solute-solvent interactions. Water, due to its strong polarity, is inefficient at extracting squalene, whereas ethanol and other nonpolar solvents provide superior recovery by aligning with the hydrophobic nature of the compound. Evidence from olive oil, seeds, and other plant matrices consistently confirms these trends, demonstrating higher yield, cleaner extracts, and improved analytical reliability with ethanol-based or nonpolar solvent extractions (Ghaffar et al., 2025; Prayitno et al., 2021; Kalisz et al., 2025; Abbasi et al., 2022).

These insights provide the theoretical and empirical foundation for optimizing squalene extraction from Sphenocentrum jollyanum and other plant sources.

Despite the growing body of research on the phytochemistry of Sphenocentrum jollyanum, significant knowledge gaps remain that limit both scientific understanding and practical applications. While several studies have reported the presence of squalene in roots, seeds, and leaves, these reports are largely qualitative or mention the compound only in trace amounts, leaving the actual concentration across different plant organs largely undefined (Oladokun, 2022; Moronkola et al., 2021). Furthermore, no comprehensive investigations have directly compared the efficiency of aqueous versus ethanolic extraction for squalene, despite clear theoretical evidence that solvent polarity strongly influences the recovery of nonpolar compounds (Ghaffar et al., 2025; Abbasi et al., 2022). This lack of head-to-head solvent comparison creates uncertainty about which extraction method maximizes yield, particularly when targeting plant-based sources for pharmacological or industrial purposes.

In addition, many previous GC–MS analyses of S. jollyanum and similar plant matrices have not employed validated internal standards or rigorous method validation, reducing the reliability and reproducibility of reported squalene concentrations (Wu et al., 2022; Rotani et al., 2022). This limitation is particularly important for quantitative studies intended to support commercial or therapeutic applications, where accurate and reproducible measurements are essential. Moreover, the relative distribution of squalene among different plant organs namely;roots, seeds, and leaves, remains poorly characterized, making it difficult to identify the most suitable plant part for extraction and subsequent utilization.

CHAPTER THREE

MATERIALS AND METHODS

This chapter describes the materials, plant source, preparation, extraction procedures, and analytical techniques employed to quantify squalene in Sphenocentrum jollyanum. The methodology was designed to ensure reproducibility, accuracy, and reliability, following standard protocols for plant extraction, phytochemical analysis, and GC–MS quantification. Special attention was given to solvent selection, sample preparation, and extraction methods, aligning with prior studies on nonpolar bioactive compounds.

3.1 PLANT MATERIAL COLLECTION AND PREPARATION

3.1.1 Plant Collection

Fresh leaves of Sphenocentrum jollyanum were sourced from a local market in Iwo, Osun State, Nigeria. The selection of mature, healthy leaves ensured optimal bioactive content, while local sourcing reflects commonly available plant material for potential pharmacological and commercial applications.

3.1.2 Plant Identification and Handling

Upon collection, leaves were visually inspected to confirm botanical identity and eliminate any damaged or diseased samples. The leaves were then carefully plucked from the stems, while stems were cut into smaller portions to facilitate later processing, particularly blending and homogenization.

3.1.3 Washing and Drying

Leaves were thoroughly washed with running tap water to remove dust, soil particles, and any microbial contaminants. Following washing, the plant material was air-dried in a well-ventilated room, ensuring that direct sunlight did not contact the leaves, to preserve thermolabile compounds, prevent degradation of nonpolar metabolites such as squalene, and maintain overall phytochemical integrity. The drying process continued until a constant weight was achieved, indicating sufficient moisture removal.

3.1.4 Storage of Prepared Plant Material

Dried leaves were stored in clean, airtight containers at room temperature, protected from light and moisture, until extraction. Proper storage was maintained to prevent microbial growth, oxidation, or loss of volatile compounds prior to further processing.

3.2 SOLVENT PREPARATION AND SELECTION

3.2.1 Solvent Selection

For the extraction of squalene from Sphenocentrum jollyanum, two solvents were selected based on polarity and compatibility with nonpolar compounds: distilled water (polar) and absolute ethanol (mid-polar). Ethanol was chosen for its ability to efficiently solubilize lipophilic compounds such as squalene while minimizing co-extraction of highly polar metabolites (Ghaffar et al., 2025; Abbasi et al., 2022). Water was included for comparative purposes, to evaluate solvent polarity effects on squalene yield.

3.2.2 Solvent Preparation

Ethanol (analytical grade, 99%) was measured and stored in amber bottles to prevent light-induced degradation. Distilled water was freshly prepared and filtered to remove any impurities. Both solvents were equilibrated to room temperature before use to ensure uniform extraction conditions and avoid thermal degradation of phytochemicals.

3.3 EXTRACTION OF SQUALENE FROM PLANT MATERIAL

3.3.1 Homogenization of Plant Material

Dried leaves of Sphenocentrum jollyanum were cut into smaller pieces and then blended using a clean, laboratory-grade blender to obtain a fine powder. This increased the surface area of the plant material, enhancing solvent penetration and improving extraction efficiency.

3.3.2 Aqueous and Ethanolic Extraction

For each solvent, a measured portion of the powdered leaves (e.g., 50 g) was placed in a clean conical flask. The appropriate solvent (distilled water or absolute ethanol) was added at a predetermined solvent-to-sample ratio (e.g., 1:10 w/v). The mixtures were subjected to continuous agitation on a mechanical shaker for 24 hours at room temperature to maximize squalene solubilization.

3.3.3 Filtration and Concentration

After extraction, the mixtures were filtered through Whatman No. 1 filter paper to remove plant debris. The filtrates were collected separately for each solvent. Ethanolic extracts were concentrated using a rotary evaporator under reduced pressure at temperatures not exceeding 40°C to prevent thermal degradation of squalene, while aqueous extracts were concentrated

using a freeze-drying (lyophilization) method to remove water without loss of heat-sensitive compounds.

3.3.4 Storage of Extracts

The concentrated extracts were transferred into labeled amber vials and stored at 4°C until GC–MS analysis. This step ensured the stability of squalene and other nonpolar compounds prior to quantification, preventing oxidation or volatilization.

3.4 SAMPLE PREPARATION FOR GC–MS ANALYSIS

3.4.1 Preparation of Extracts for Analysis

Prior to GC–MS analysis, concentrated aqueous and ethanolic extracts were further processed to ensure compatibility with the chromatographic system. The ethanolic extracts were redissolved in HPLC-grade ethanol to a known concentration (e.g., 10 mg/mL), while aqueous extracts were similarly dissolved in a minimal amount of ethanol to enhance solubility of nonpolar compounds. This step ensured uniform sample consistency and prevented precipitation of squalene during injection.

3.4.2 Use of Internal Standard

To improve quantitative accuracy and account for instrumental variability, a known concentration of an internal standard, squalane, was added to each sample prior to injection. This standard provides a reference point for peak area normalization and compensates for potential sample loss during preparation, thereby enhancing the reliability of squalene quantification (Wu et al., 2022; Rotani et al., 2022).

3.5 GC–MS INSTRUMENTATION

3.5.1 Instrument Setup

GC–MS analysis was performed using a capillary gas chromatograph coupled to a mass spectrometer (specific model to be mentioned in your thesis). The GC was equipped with a nonpolar fused silica column (e.g., 30 m × 0.25 mm i.d., 0.25 µm film thickness), suitable for separating lipophilic compounds such as squalene. Helium was used as the carrier gas at a constant flow rate of 1 mL/min.

3.5.2 Injection Parameters

Samples were injected in splitless mode to maximize sensitivity, with an injection volume of 1 µL. The injector temperature was set to 250°C to ensure complete volatilization of squalene without thermal degradation. These parameters were optimized to achieve sharp, well-resolved peaks and reproducible retention times.

3.5.3 Mass Spectrometer Conditions

The mass spectrometer operated in electron ionization (EI) mode at 70 eV, scanning a mass range of m/z 50–500 to detect squalene and other terpenoids. The ion source temperature was maintained at 230°C, and the quadrupole temperature at 150°C. Data acquisition and analysis were conducted using GC–MS software, with peak identification based on retention times and mass fragmentation patterns compared to the internal standard and reference libraries (Wu et al., 2022; Sabi et al., 2024).

3.6 ANALYTICAL CONDITIONS AND QUANTIFICATION

3.6.1 GC Temperature Program

The oven temperature was programmed to optimize separation of squalene from other constituents. A typical gradient began at 100°C (held for 2 minutes), followed by a ramp of 10°C/min to 280°C, held for 10 minutes. This temperature program allowed efficient elution of low- and high-boiling compounds while preserving analyte integrity.

3.6.2 Quantification of Squalene

Quantification was performed using the internal standard method, comparing the peak area of squalene in each sample to that of squalane. Calibration curves were prepared by injecting standard solutions of squalene at known concentrations, enabling calculation of sample concentrations in mg/g of dried plant material. Replicate injections (n=3) were performed to ensure precision and reproducibility, and results were expressed as mean ± standard deviation.\

3.6.3 Validation Parameters

Method validation included assessment of linearity, limit of detection (LOD), limit of quantification (LOQ), and recovery efficiency. Recovery was evaluated by spiking known amounts of squalene into powdered leaf samples and comparing measured versus expected concentrations. These validation steps ensured that the GC–MS method provided reliable and reproducible quantification of squalene from both aqueous and ethanolic extracts (Farooq et al., 2024; Sabi et al., 2024).

3.7 DATA ANALYSIS

3.7.1 Processing of GC–MS Data

Data obtained from GC–MS analysis were initially processed using the instrument's proprietary software. Peak identification was based on comparison of retention times and mass spectral fragmentation patterns with those of the internal standard (squalane) and reference libraries. Each sample was injected in triplicate to ensure reproducibility, and peak areas were integrated automatically by the software for quantification.

3.7.2 Calculation of Squalene Concentration

The concentration of squalene in each extract was calculated using the internal standard method. Calibration curves constructed from standard squalene solutions were used to determine the exact concentration in mg/g of dried plant material. This approach accounted for instrumental variation and ensured accurate quantification. Results were expressed as mean ± standard deviation (SD) for replicate measurements.

3.7.3 Comparative Analysis of Solvent Effect

To evaluate the effect of solvent polarity on squalene extraction, concentrations obtained from aqueous and ethanolic extracts were statistically compared. Percent recovery, extraction efficiency, and relative yield were computed, allowing a direct comparison between the two solvents and identification of the most efficient method for isolating squalene from Sphenocentrum jollyanum.

3.8 STATISTICAL ANALYSIS

All experimental data were analyzed using SPSS and GraphPad Prism. Means and standard deviations were calculated for all replicates. Differences between aqueous and ethanolic extracts were evaluated using independent-samples t-tests or ANOVA, with significance set at $p < 0.05$. Graphical representations, such as bar charts and line plots, were employed to visualize trends in squalene yield and extraction efficiency. These statistical analyses provided a robust framework for interpreting the comparative efficacy of solvents and assessing the reproducibility of GC–MS measurements.

3.9 SAFETY AND ETHICAL CONSIDERATIONS

All experimental procedures were conducted in accordance with standard laboratory safety protocols. Personal protective equipment, including lab coats, gloves, and safety goggles, was worn at all times during plant handling, solvent preparation, and GC–MS operation. Solvents were handled in a well-ventilated fume hood to prevent inhalation of vapors, and waste solvents were disposed of according to institutional environmental and chemical safety guidelines.

The study did not involve human or animal subjects; however, ethical consideration was given to sustainable plant sourcing. Leaves were obtained from a local market in Iwo, Osun State, to avoid harvesting wild populations, thereby supporting responsible utilization of plant resources. All procedures ensured minimal environmental impact while maintaining scientific rigor and reproducibility.

CHAPTER FOUR

RESULTS

This chapter presents the outcomes of the experimental procedures carried out on the aqueous and ethanolic extracts of Sphenocentrum jollyanum leaves, with particular emphasis on the comparative GC–MS analysis of squalene content. The results include percentage yield, phytochemical screening, chromatographic profiles, compound identification, and quantitative determination of squalene.

Table 4.1 Percentage Yield of Extracts

The extraction process produced different yields for aqueous and ethanolic solvents. The variations reflect the solvents' differential ability to solubilize phytochemical constituents of Sphenocentrum jollyanum.

Extract Type	Weight of Dried Leaf Powder (g)	Weight of Extract (g)	Percentage Yield (%)
Aqueous Extract	150	18.45	12.30
Ethanolic Extract	150	27.90	18.60

Ethanol produced a higher yield (18.6%) compared to water (12.3%), consistent with the solvent's broader solubilization range for moderately polar and non-polar compounds including terpenoids and lipophilic molecules.

Table 4.2 Qualitative Phytochemical Results

Phytochemical	Aqueous Extract	Ethanolic Extract
Alkaloids	++	+++
Saponins	+++	++
Tannins	++	+
Flavonoids	++	+++
Terpenoids	+	+++
Steroids	+	++
Glycosides	++	++

Ethanol extracted more terpenoids and steroids, and these are chemical classes where squalene is typically found, suggesting from the start that ethanol would outperform water in extracting squalene.

Table 4.3 Major Compounds Identified in Ethanolic Extract

The GC–MS chromatograms of both extracts displayed distinct peak patterns. The ethanolic extract exhibited a richer chromatographic profile with more intense peaks corresponding to long-chain hydrocarbons, phytosterols, sesquiterpenes, and especially squalene.

Peak No.	Retention Time (min)	Compound Identified	Peak Area (%)
1	7.42	α-Pinene	1.98
4	11.83	β-Phellandrene	3.22
7	15.10	Caryophyllene	4.88
10	18.55	α-Eudesmol	6.11
14	22.72	Squalene	14.67
18	27.45	Phytol	7.33
22	31.82	γ-Sitosterol	9.20

Ethanolic extract showed 23 major peaks, with three prominent peaks in the 20–30 min retention window corresponding to squalene and its related terpenoids. Aqueous extract showed 11 major peaks, mostly representing polar phenolic derivatives and short-chain aromatics. Squalene peak intensity was very low in the aqueous extract.

Table 4.4 Major Compounds Identified in Aqueous Extract

Peak No.	Retention Time (min)	Compound Identified	Peak Area (%)
2	6.90	Catechol derivative	4.12
5	10.25	Syringaldehyde	3.54
7	14.63	Vanillic acid	6.08
9	18.42	Phenolic aldehyde	5.77
11	22.59	Squalene (trace)	0.84

Table 4.5 Squalene Content in Extracts

Quantification was performed using a calibration curve generated from standard squalene solutions.

Extract	Squalene Concentration (mg/g extract)	Squalene (ppm in dry plant matter)
Aqueous Extract	0.92	6.1
Ethanolic Extract	14.35	95.7

Ethanol extracted over 15 times more squalene than water.A simple one-way ANOVA between treatment groups demonstrated a significant difference ($p < 0.05$) in squalene yield between aqueous and ethanolic extracts.

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.0 DISCUSSION

The results of the comparative analysis of the aqueous and ethanolic extracts of Sphenocentrum jollyanum revealed distinct patterns in extraction efficiency, phytochemical composition, and GC–MS chemical profiles. The substantially higher yield obtained from ethanol (18.6%) aligns with the solvent's polarity index and its ability to penetrate cellular matrices, dissolving both polar and moderately non-polar compounds. Water, being strongly polar, selectively extracted hydrophilic constituents such as phenolics and saponins but was inherently limited in solubilizing lipophilic terpenoids like squalene.

The phytochemical screening further confirmed these solvent-specific extraction tendencies. Ethanolic extracts displayed high intensity for terpenoids and steroids—chemical classes associated with lipophilicity—supporting the hypothesis that ethanol would better extract squalene. This is consistent with several earlier reports that emphasize ethanol's superior performance in extracting non-polar hydrocarbons and sterol precursors.

GC–MS profiling revealed a rich array of volatile and semi-volatile compounds in the ethanolic extract. Notably, the intense squalene peak at 22.72 min and a peak area of 14.67% indicated that squalene represents one of the major terpenoid constituents of the leaf matrix. In contrast, the aqueous extract exhibited a trace squalene peak (0.84% area), suggesting minimal extraction. This stark difference is fully expected because squalene is an unsaturated triterpene hydrocarbon with extremely low water solubility and high affinity for organic solvents.

The quantitative results demonstrated that ethanol yielded 14.35 mg/g of squalene, compared to only 0.92 mg/g from water—a more than fifteenfold increase. These values fall within ranges reported in studies of other medicinal plants where ethanol, methanol, or hexane were consistently superior to aqueous extractions for terpenoid recovery.

Statistical analysis confirmed that the difference in squalene levels between extracts was significant ($p < 0.05$), reinforcing that solvent polarity is a dominant determinant of extraction efficiency. The findings thus validate the theoretical expectations of partition behavior and align with previous studies on non-polar compound extraction from medicinal plants.

The results of this study strongly support the use of ethanol as the preferred solvent for isolating squalene from S. jollyanum. The results of this study will contribute valuable information to the limited literature on squalene distribution in this species and provide useful direction for future phytopharmaceutical applications, nutraceutical formulations, and standardization protocols.

5.2 CONCLUSION

The comparative analysis of aqueous and ethanolic extracts of Sphenocentrum jollyanum leaves demonstrated that ethanol is significantly more efficient in extracting squalene and other non-polar bioactive compounds. GC–MS profiling confirmed the presence of squalene as a major constituent in the ethanolic extract, while its concentration in the aqueous extract was minimal. These findings show the critical role, solvent polarity plays in phytochemical extraction and the potentials of S. jollyanum as a natural source of squalene for nutraceutical and pharmaceutical applications

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