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# Phytochemical analysis of *Leptadenia madagascariensis* Decne (Apocynaceae)

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***Abstract:***

*Leptadenia madagascariensis Decne, a woody climbing vine endemic to Madagascar, has long been utilized in traditional medicine for its therapeutic properties, despite a lack of detailed scientific investigation. This study was conducted to perform a comprehensive phytochemical analysis of the plant's aerial parts, thereby contributing to the chemical validation of its ethnobotanical applications and supporting national conservation efforts. The powdered plant material was successively macerated with hexane, dichloromethane (DCM), and methanol, yielding crude extracts with varying polarity. Phytochemical screening of these extracts revealed a significant concentration of polysaccharides, saponins, and steroids. The DCM extract, showing a promising phytochemical profile, was selected for further purification. An initial analysis by thin-layer chromatography (TLC) indicated the presence of four dominant compounds. Subsequent separation using column chromatography resulted in the isolation of a pure compound from fractions 55 to 70. The structure of this isolated compound was determined through a combination of spectroscopic techniques, including mass spectrometry and one-dimensional nuclear magnetic resonance (NMR) spectroscopy (1H NMR, 13C NMR, and DEPT). The spectral data were compared with literature values, leading to the definitive identification of the molecule as β-Sitosterol, a common tetracyclic steroid. This finding confirms the presence of therapeutically valuable steroid compounds in L. madagascariensis, providing a scientific basis for its traditional use and a starting point for further pharmacological research.*

***Keywords:***
*Leptadenia madagascariensis, phytochemical analysis, β-Sitosterol, chromatography, NMR spectroscopy*

## I. Introduction

Madagascar, a global biodiversity hotspot, harbors a rich array of endemic plant species with significant ethnobotanical importance. Among these is *Leptadenia madagascariensis* Decne, a woody, climbing vine indigenous to the island and a member of the Apocynaceae family (**Liede & Weber, 2017**). The plant is integral to the traditional medicine of the Toliara province, where different parts are prepared as decoctions to treat a variety of human and animal ailments, including postpartum care, lactation support, constipation, and general fatigue (**Jardins du Monde, 2016**). This targeted use highlights a sophisticated body of local knowledge concerning the species' therapeutic properties. The botanical classification of the plant and its distinction from other *Leptadenia* species have also been a subject of recent taxonomic investigation (**Judd et *al.*, 2016 ; Augros & Hoareau, 2018**).

Despite its traditional use, a detailed phytochemical profile of *L. madagascariensis* has not been extensively documented. The primary objective of this study was to contribute to the chemical understanding of this plant, thereby supporting the national effort to conserve medicinal flora and validate local traditional knowledge. The research employed a systematic approach, beginning with the successive maceration of the plant's aerial parts using solvents of increasing polarity (**Harborne, 1998 ; Nascimento et *al.*, 2021**). Subsequent phytochemical screening was performed to identify major classes of secondary metabolites, followed by chromatographic techniques for the isolation and purification of a target compound (**Skoog et *al.*, 2017**). The structural elucidation of the isolated product was achieved through advanced spectroscopic methods, including mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy. This comprehensive investigation aimed to chemically validate the therapeutic potential of *L. madagascariensis* as a medicinal resource.

## II. Research Method

### 2.1 Plant material: Botanical description of *Leptadenia madagascariensis* Decne

*Leptadenia madagascariensis*, an Apocynaceae family member (subfamily Asclepiadoideae), is a woody, climbing vine indigenous to Madagascar (**Liede & Weber, 2017**). The plant features a cylindrical, grayish-brown stem that can extend up to 10 meters in length and 8 cm in diameter, containing an abundant white latex. Foliage is simple and oppositely arranged, with leaves that are elliptic to ovate, measuring 4-12 cm long and 2-8 cm wide, distinguished by an acuminate apex and a rounded base (**Judd et *al.*, 2016**). Petioles are 1-3 cm in length.

Floral structures consist of small, greenish to pale yellow flowers organized in axillary or terminal cymes. The calyx is five-lobed, and the corolla is composed of five basally fused, twisted lobes. The five stamens are inserted at the base of the corolla, with anthers fused around the style. The fruit is a dehiscent, spindle-shaped follicle, 8-15 cm long and 1-2 cm in diameter. These brown follicles contain numerous flat, oval, brownish seeds, each 5-7 mm long, which are crowned by a tuft of silky hairs (**Stevens, 2017**). Flowering occurs from November to April, with fruiting following from December to May.

### 2.2 Uses of *Leptadenia madagascariensis* Decne in traditional medicine in the province of Toliara

Within the Toliara province of Madagascar, *Leptadenia madagascariensis* is utilized in traditional medicine to address a variety of human and animal ailments. This species, an indigenous woody vine, is integral to local ethnobotanical practices, with distinct plant parts and preparation methods tailored to specific conditions (**Jardins du Monde, 2016**).

For maternal and postpartum care, a decoction derived from the plant's roots is administered both internally and externally. This preparation is believed to facilitate childbirth and aid in the mother's recovery. Furthermore, the consumption of a decoction made from the plant's latex or leaves is used to stimulate lactation, supporting breastfeeding mothers. The entire plant is also prepared as a decoction to alleviate common discomforts such as constipation and general fatigue.

In addition to its human applications, the plant serves in local veterinary care. A preparation from the leafy stems is applied topically around the horns of zebu cattle. This practice is specifically used to prevent a condition known as "*kady*" or to treat "*beravigny*," a malady characterized by drooping ears and a loss of appetite in the animals. The targeted use of different plant parts and specific preparation methods underscores a sophisticated body of

traditional knowledge regarding the therapeutic properties of this species (**Augros & Hoareau, 2018**).

### 2.3 Extraction methods

Extraction is a fundamental technique in chemical engineering and chemistry, designed to selectively separate one or more compounds from a mixture based on their unique chemical or physical properties (**Zeb et *al.*, 2021**). These methods are generally classified into two main types: solid-liquid extraction and liquid-liquid extraction.

#### a. Solid-liquid extraction

Solid-liquid extraction is a common method for isolating chemical compounds from solid matrices, such as plant materials, foods, or environmental samples, using a liquid solvent. This process exploits the differences in compound solubility to achieve separation from the solid material. Various techniques can be employed, including maceration, infusion, decoction, and Soxhlet extraction (**Azwanida, 2015**). For phytochemical analysis, a particularly effective approach is successive maceration using solvents of increasing polarity.

The process begins with the preparation of the solid sample. Harvested plant leaves are dried in a well-ventilated area, away from direct sunlight and humidity. The dried leaves and stems are then ground to a fine powder to increase the surface area, facilitating efficient solvent penetration. In successive maceration, the prepared plant material is subjected to a series of extractions using solvents in order of increasing polarity, such as hexane, followed by dichloromethane (DCM), and finally methanol. Each maceration period typically lasts for about 48 hours. The resulting heterogeneous mixture is filtered using hydrophilic cotton to separate the liquid extract from the solid residue. To recover the extracted compounds, the solvent is then gently removed from each filtrate using a rotary evaporator, which operates under a moderate temperature and reduced pressure to prevent the degradation of heat-sensitive compounds (**Zeb et *al.*, 2021**). This systematic method ensures the isolation of a wide range of compounds with varying polarities.

#### b. Liquid-liquid extraction

Liquid-liquid extraction is a key separation technique used to isolate compounds from a plant extract by partitioning them between two immiscible solvents, typically an aqueous phase and an organic solvent (**Skoog et *al.*, 2017**). This method capitalizes on the differential solubility of compounds based on their polarity. The aqueous solvent preferentially dissolves polar constituents, while the organic solvent, which is moderately polar or non-polar, selectively extracts the non-polar and slightly polar compounds (**Pawliszyn, 2018**). The process effectively separates a complex mixture into distinct fractions, each enriched with compounds of a specific polarity range, enabling targeted phytochemical analysis.

#### c. Phytochemical screening

Phytochemical screening serves as the initial step in the chemical analysis of plant materials, employing a series of characteristic tests to identify major compound classes (**Nascimento et *al.*, 2021**). This qualitative approach aims to detect the presence of various secondary metabolites, including alkaloids, coumarins, flavonoids, polyphenols, polysaccharides, quinones, saponins, steroids, tannins, and terpenoids. Generally, an hydroalcoholic extract is prepared from the powdered plant material using 80% ethanol, with subsequent detection relying on either colorimetric or precipitation tests.

- The **alkaloid** screening involves macerating a dried plant powder sample in 10% HCl. The resulting filtrate is then subjected to specific precipitating agents. A positive result is

- indicated by the formation of a precipitate: a yellowish-white precipitate with Wagner's reagent, an orange precipitate with Mayer's reagent, and an orange-red precipitate with Dragendorff's reagent.
- For **coumarins**, the hydroalcoholic extract is spotted onto paper and examined under UV light at 365 nm. A positive result is confirmed by a characteristic yellow fluorescence around the spot (**Cowan, 1999**).
- **Flavonoid** screening utilizes the Wilstater and Bath-Smith tests. The extract is first washed with hexane to remove pigments, then dissolved in ethanol. The Wilstater test, involving the addition of concentrated HCl and magnesium turnings, results in a red, purple, or reddish-violet color, indicating the presence of flavans, flavanols, or flavonones, respectively. The modified Wilstater test, which includes an additional phase separation step with isoamyl alcohol, yields a red or purple color in the upper layer, signaling the presence of flavones or flavonols. The Bath-Smith test, which involves heating with concentrated HCl, produces a reddish-violet color for leucoanthocyanins or a red color for anthocyanins.
- **Polyphenols and tannins** are detected in a filtered hydroalcoholic extract. A precipitate formed with 1% gelatin indicates polyphenols, while a precipitate with salted gelatin specifically points to tannins. The presence of condensed tannins is shown by a blue-green color with 10% $FeCl_3$, whereas hydrolyzable tannins produce a bluish-black coloration (**Harborne, 1998**).
- The presence of **polysaccharides** is confirmed when a decoction of the plant powder, upon the addition of ethanol, forms a precipitate.
- **Saponins** are identified through a frothing test: a plant powder and distilled water mixture, when vigorously shaken, produces a stable foam of at least 3 cm in height.
- **Quinones** are screened by extracting the hydroalcoholic extract with a petroleum ether/chloroform mixture. A reddish-violet coloration in the alkaline phase upon the addition of NH4OH confirms their presence.
- Finally, **steroids and terpenoids** are screened using an extract partitioned into chloroform. The Lieberman-Burchard test produces a purple color for terpenoids or a violet/blue-green color for steroids. The Salkowski test shows a red ring at the interface of the chloroform and concentrated $H_2SO_4$ layers, indicating unsaturated sterols, while the Badget-kedde test yields a red color for lactonic steroids.

**d. Chromatographic analysis methods**

a) **Thin-layer chromatography (TLC)**

Thin-layer chromatography (TLC) is a widely used biochemical technique for separating and identifying the components of a mixture (**Sherma & Fried, 2011**). The principle relies on the differential partitioning of compounds between two phases: a stationary phase and a mobile phase. The stationary phase, typically a thin layer of an adsorbent like silica gel on a rigid backing such as an aluminum sheet or glass plate, provides a surface for the separation. The mobile phase, or eluent, is a solvent or solvent mixture that moves up the plate by capillary action.

The separation process is driven by adsorption phenomena, where compounds in the mixture migrate at varying speeds based on their chemical nature and interactions with both the stationary and mobile phases. In normal-phase TLC, less polar substances migrate more rapidly than more polar components due to weaker interactions with the polar stationary phase (**Skoog et *al.*, 2017**).

The main components of a TLC setup include a chromatographic chamber, a TLC plate, the sample, and the eluent. The chromatographic chamber, a sealed glass container, is

first saturated with the solvent vapor to ensure consistent migration. A small, concentrated spot of the sample is then applied to a carefully drawn baseline on the plate using a micropipette or capillary. The plate is then placed vertically into the chamber, ensuring the solvent level is below the sample spots.

As the eluent ascends the plate, it carries the sample components with it. Once the solvent front reaches a predetermined height, the plate is removed and dried. The separated compounds, which are often colorless, are then visualized using one of two primary methods. A physical method involves examination under ultraviolet (UV) light at wavelengths of 254 nm or 365 nm, where compounds with chromophores or fluorophores become visible (**Lazarovici et *al.*, 2018**). Alternatively, a chemical method, such as spraying with a reagent like anisaldehyde, followed by heating, causes a color-forming reaction that reveals the separated spots. This two-step process of elution and revelation provides a powerful tool for qualitative analysis.

b) **Column chromatography (CC)**

Column chromatography is a widely used preparatory technique for the separation and isolation of compounds from a mixture, capable of handling sample sizes from milligrams to several grams in a laboratory setting (**Snyder et *al.*, 2010**). This method is based on adsorption phenomena, where components of a mixture are separated as they move through a stationary phase packed inside a vertical column. The process, while powerful, can be empirical and often requires optimization through trial and error.

The operation involves several key steps: preparing the gel, packing the column, equilibrating it, introducing the sample, performing elution, collecting fractions, and analyzing the resulting chromatogram. The most critical step is packing the column, which must be done to ensure a uniform bed without air bubbles to achieve optimal separation. Two primary methods for packing are the dry and wet-packing techniques.

In dry packing, the column is filled two-thirds with the least polar solvent, and a powdered adsorbent is gradually added while tapping the column walls to ensure maximum compaction. The solvent is then allowed to drain slowly as more adsorbent is added. Wet packing, a more common method, involves preparing a homogeneous slurry of the adsorbent in the least polar solvent. This slurry is then poured into the column, allowing the adsorbent to settle and form a uniform layer as the solvent drains. The process is continued until the desired bed height is reached.

Elution, the process of passing the mobile phase through the column, is often performed using a gradient elution method. This involves progressively increasing the polarity of the mobile phase to sequentially elute compounds based on their affinity for the stationary phase. Typical solvent systems for this process include dichloromethane (DCM) alone, or mixtures of DCM and methanol in increasing proportions, such as 99:1 and 98:2 (DCM/MeOH, v/v).

**e. Nuclear Magnetic Resonance spectroscopy**

**a) Proton Nuclear Magnetic Resonance ($^1$H NMR) spectroscopy**

Following chromatographic separation, spectroscopic analysis, particularly Proton Nuclear Magnetic Resonance ($^1$H NMR) spectroscopy, is a powerful tool for structural elucidation of the isolated compounds (**Skoog et *al.*, 2017**). This technique provides crucial information about a molecule's structure through several key parameters:

- **Chemical Shift (δ)**: The position of a signal, measured in parts per million (ppm), indicates the electronic environment of a proton. Protons in chemically equivalent environments exhibit the same chemical shift.
- **Number of Signals:** This corresponds to the number of chemically non-equivalent proton groups in the molecule.
- **Signal Multiplicity:** Governed by the (n+1) rule, this refers to the splitting of a signal into multiple peaks (a multiplet), which is determined by the number of neighboring protons (n) on adjacent atoms.
- **Signal Integration:** The area under a signal's curve is directly proportional to the number of protons responsible for that signal, providing a ratio of protons in different chemical environments.

**b) Carbon-13 Nuclear Magnetic Resonance ($^{13}$C NMR) Spectroscopy**

$^{13}$C NMR Spectroscopy is a fundamental tool for determining the carbon backbone of organic molecules. Its analysis is based on the chemical shifts of carbon atoms, which provide information about their unique electronic environments (**Skoog et *al.*, 2017**). A key technique in this field is Distortionless Enhancement by Polarization Transfer (DEPT), which differentiates carbon signals based on the number of attached protons. The DEPT experiment uses multi-pulse sequences to transfer polarization from protons to carbons, making it possible to distinguish between primary ($CH_3$), secondary ($CH_2$), tertiary (CH), and quaternary (C) carbons, which are typically invisible in these spectra (**Hornak, 2010**).

The results of a DEPT analysis are interpreted from three distinct spectra, each generated by a different pulse angle (θ) :

- DEPT-45 (θ= 45°): This spectrum shows positive signals for all carbons with attached protons ($CH_3$, $CH_2$, CH).
- DEPT-90 (θ= 90°): This spectrum provides a simplified view, displaying only positive signals for tertiary carbons (CH).
- DEPT-135 (θ= 135°): This is the most informative DEPT spectrum, showing positive signals for $CH_3$ and CH groups, while simultaneously presenting negative signals for $CH_2$ groups

Complementing the DEPT experiment is the proton-decoupled $^{13}$C NMR spectrum, often referred to as a broadband decoupled spectrum. This technique simplifies the spectrum by collapsing all proton-carbon coupling interactions into single, sharp peaks. This is achieved by irradiating the sample with a broad range of radio frequencies that cover the entire proton resonance domain (**Pavia et *al.*, 2015**). As a result, the number of observed signals directly corresponds to the number of chemically non-equivalent carbon atoms in the molecule, assuming no signal overlap. The intensity of these signals is also proportional to the number of protons attached to each carbon, making them valuable for quick structural estimations.

## III. Result and Discussion

### 3.1 Extraction results

Following preliminary air-drying and grinding, 28 grams of the aerial parts of *Leptadenia madagascariensis* were subjected to sequential maceration. A quantity of 250 mL of each solvent—hexane, dichloromethane (DCM), and methanol (MeOH)—was used in succession over a 48-hour period to obtain crude extracts based on increasing polarity. The extracts were subsequently filtered and evaporated using a rotary evaporator. The mass and yield of each crude extract were as follows: hexane extract at 1.1 g (3.93% yield), DCM extract at 0.88 g

(3.14% yield), and MeOH extract at 0.64 g (2.29% yield). These extracts were then used for phytochemical screening and chromatographic analysis.

### 3.2 Phytochemical screening results

The phytochemical analysis of the leaf powder revealed the absence of several major secondary metabolite classes. Tests for alkaloids using Mayer's, Wagner's, and Dragendorff's reagents all yielded negative results, as indicated by a lack of precipitation. Similarly, the screening for coumarins showed no yellow fluorescence under UV light, and tests for flavonoids, polyphenols, and tannins also came back negative, with no observed color change or precipitation. The quinones test also showed no color change, suggesting their absence or presence in a form undetectable by the assay (**Evans, 2019**). The data consistently indicates that the leaf material is devoid of these compounds.

***Table 1.*** *Phytochemical screening results*

| **Chemical families** | **Part studied** | **Results** |
|---|---|---|
| Alkaloids | Leaf | **-** |
| Coumarins | Leaf | **-** |
| Flavonoids | Leaf | **-** |
| Polyphenols | Leaf | **-** |
| Tannins | Leaf | **-** |
| Quinones | Leaf | **-** |
| **Polysaccharides** | Leaf | **+++** |
| **Saponins** | Leaf | **+** |
| **Steroids** | Leaf | +++ |
| Terpenoids | Leaf | - |

In contrast, several key compound classes were positively identified. The saponin test produced a 2 cm foam height, indicating a weak presence (+). A decoction of the leaf material formed a strong precipitate upon the addition of ethanol, confirming a high concentration of polysaccharides (+++). Furthermore, a comprehensive screening for steroids and terpenoids yielded interesting results. The Lieberman-Burchard and Salkowski tests indicated a weak to moderate presence of steroids (+ to ++), while the Badget-Kedde test showed an intense color change (+++), suggesting a substantial presence of lactonic steroids. The terpenoid test, however, was negative. The overall phytochemical profile of the aerial parts of *Leptadenia madagascariensis* is characterized by the abundance of polysaccharides and steroids, with a moderate presence of saponins and a notable absence of alkaloids, coumarins, flavonoids, polyphenols, tannins, quinones, and terpenoids.

### 3.3 Chromatography analysis

Both thin-layer chromatography (TLC) and column chromatography were employed for the analysis of extracts from the aerial parts of *Leptadenia madagascariensis*. The dichloromethane (DCM) extract was specifically chosen for chromatographic analysis due to its sufficient quantity and prior research indicating its suitability for separation using various solvent systems (**Harborne, 1998; Sherma & Fried, 2011**).

#### a. zzzzzzzzzzzzzThin-Layer Chromatography (TLC) results

The DCM extract was applied to a TLC plate with a 1 cm offset from the bottom edge, and the solvent migration was halted when the front reached 1 cm from the top. Initial tests were conducted with pure hexane, a hexane/DCM mixture (50/50), pure DCM, and a DCM/methanol (MeOH) mixture (98/02). The developed chromatograms were visualized

under UV light at 254 nm and 366 nm, followed by a chemical spray using 10% sulfuric acid in methanol and subsequent heating at 120 °C.

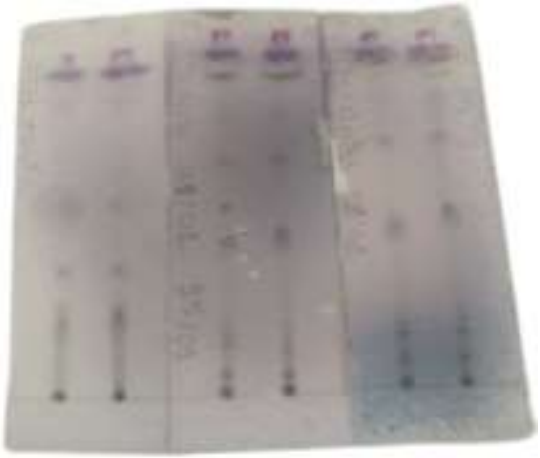


**Figure 1.** Chromatographic analysis of DCM extract

The chromatogram developed with pure DCM showed poor separation, appearing as a single, smeared spot. This "streaking" indicates that the solvent system was not effective in resolving the components, likely due to the varied polarities of the compounds within the extract (**Skoog et *al.*, 2017**). Conversely, the chromatograms from the DCM/MeOH systems (99/01 and 98/02) demonstrated improved separation, showing distinct spots. However, some spots still overlapped, suggesting that further optimization of the mobile phase is necessary to achieve complete resolution of the individual compounds. The initial TLC results confirm the presence of multiple secondary metabolites in the DCM extract, particularly steroids and polysaccharides, as indicated by the prior phytochemical screenings.

**b. Column Chromatography (CC) results**

Based on the results from thin-layer chromatography (TLC), the dichloromethane (DCM) extract was selected for column chromatography to achieve further separation and purification. This preparatory technique was conducted using a cylindrical Pyrex glass column measuring 15 cm in height and 2 cm in inner diameter.

The column was meticulously packed with a thick slurry of silica gel, resulting in a stationary phase height of 42.5 cm. The sample was carefully loaded onto the top of the column bed, and elution commenced using pure DCM as the mobile phase. A total volume of approximately 800 mL of eluent was passed through the column to ensure complete separation of the components. This process yielded 102 distinct fractions, which were collected sequentially for subsequent analysis (**Skoog et *al.*, 2017**). The choice of a single solvent system, rather than a gradient, suggests a focus on isolating less polar or moderately polar compounds that are soluble in DCM. This methodical approach is critical for the isolation and purification of individual compounds from complex plant extracts, paving the way for their structural elucidation (**Sherma & Fried, 2011**).

The combined results from thin-layer chromatography (TLC) and column chromatography provided a detailed understanding of the DCM extract's composition. This analytical process not only identified an optimal solvent system for separating the extract's components but also corroborated the findings from the initial phytochemical screening (**Harborne, 1998**). The chromatographic analysis of all collected fractions from the column separation revealed that fractions 55 to 70 contained a single, pure compound.

This particular fraction, designated F55-70, yielded 9.8 mg of a purified product. The successful isolation of a single compound in sufficient quantity for subsequent analysis is a significant achievement. This purified product will be used for further structural elucidation, likely through advanced spectroscopic techniques such as NMR and Mass Spectrometry. This systematic approach—from initial screening and preliminary separation to targeted purification—underscores the logical progression of phytochemical research, ensuring that the isolated compounds are of high purity for accurate structural determination (**Skoog et *al.*, 2017**).

### 3.3 Determination of isolated product structure

Analytical techniques such as mass spectrometry (MS) and Nuclear Magnetic Resonance (NMR) spectroscopy are fundamental for the structural elucidation of isolated compounds. Mass spectrometry is an exceptionally sensitive method for detecting and identifying molecules by determining their molecular weight with high precision.

#### a. Mass spectrometry (MS)

To obtain an electron impact (EI) mass spectrum, a mass spectrometer, such as the FINNIGAN MAT 312 model, is used. The sample is introduced and subjected to an electron bombardment source at a specific energy, typically 70 eV. This process converts the neutral molecules into positively charged radical ions, known as molecular ions (M+), which subsequently fragment into smaller, characteristic ions. The instrument then separates these ions based on their mass-to-charge ratio (m/z) and records their abundance (**Silverstein et *al.*, 2014**). The resulting spectrum provides crucial information, with the highest m/z peak often corresponding to the molecular ion, which is vital for the precise determination of the compound's molecular weight (**McLafferty & Tureček, 1993**). This detailed information on molecular mass and fragmentation patterns is essential for confirming the identity and purity of the isolated product.

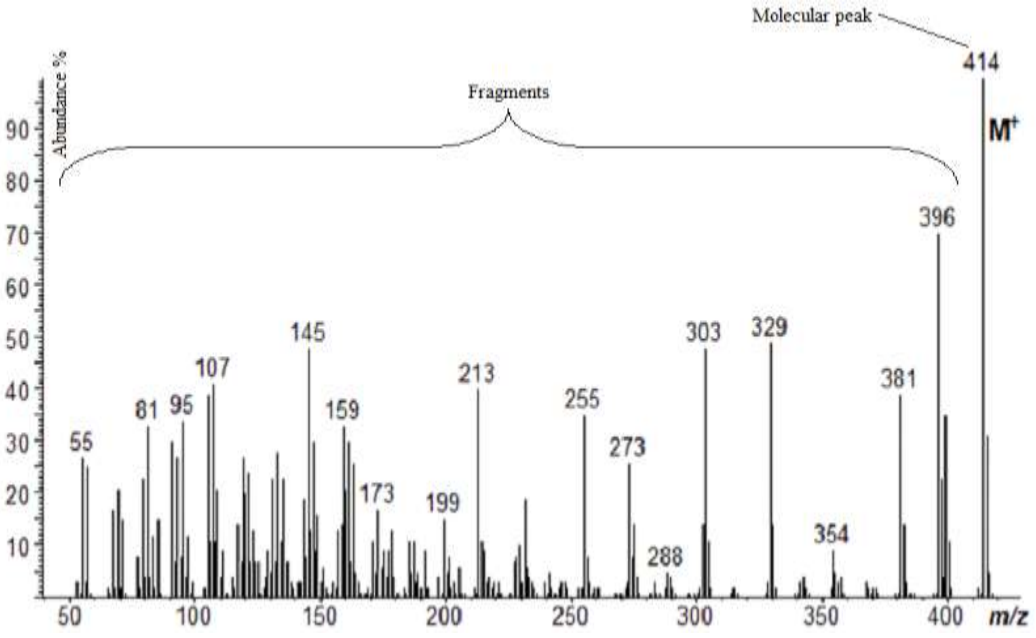



**Figure 2**. EI-MS mass spectrum of isolated product

The mass spectrum analysis indicates that the molecular ion peak has an m/z value of 414, which corresponds to the molecular mass of the isolated compound. Fragmentation peaks at lower m/z values provide clues to the compound's structure.

**Fragmentation Analysis**

The observed fragmentation patterns suggest the loss of specific neutral molecules or radicals from the parent ion. The presence of a fragment ion at m/z = 396 indicates a loss of 18 mass units, which is characteristic of the elimination of a water molecule ($H_2O$). This loss is a common fragmentation pathway for compounds containing hydroxyl groups, such as alcohols (**Skoog et *al.*, 2017**).

Furthermore, a fragment ion at m/z = 381 signifies a loss of 33 mass units from the molecular ion. This loss can be interpreted as the combined elimination of a water molecule ($H_2O$) and a methyl radical ($CH_3$), as 18 + 15 equals 33. The sequential or concerted loss of these two groups provides insight into the presence of both a hydroxyl group and a methyl group in the molecule (**Harborne, 1998**). The analysis of these fragmentation patterns allows for the partial reconstruction of the compound's structural components.

**b. NMR spectroscopy**

Finalizing the structural determination of the isolated compound involved the use of Nuclear Magnetic Resonance (NMR) spectroscopy, a powerful technique based on the magnetic properties of atomic nuclei (**Skoog et *al.*, 2017**). This method measures the absorption of radiofrequency radiation by a nucleus in a strong magnetic field. The NMR spectra were recorded at the Institute of Organic Chemistry at the University of Hanover, Germany, using a Bruker AV 400 spectrometer. This instrument operates at 400 MHz for proton ($^1H$) NMR and 100 MHz for carbon-13 ($^{13}C$) NMR. For these analyses, the compound was dissolved in deuterated chloroform ($CDCl_3$).

- **$^1H$ NMR Spectra**

The position of signals in a $^1H$ NMR spectrum is measured as a chemical shift (δ), which is expressed in parts per million (ppm). This dimensionless value is a measure of a nucleus's resonance frequency relative to a standard internal reference, typically tetramethylsilane (TMS), which is assigned a chemical shift of 0 ppm (**Pavia et *al.*, 2015**). A signal's position on the spectrum provides key information about the electronic environment of the protons. Signals that appear at higher ppm values (further to the left) are considered "deshielded," indicating that the protons are in an electron-poor environment, such as near an electronegative atom. Conversely, signals that appear at lower ppm values (further to the right) are "shielded," suggesting the protons are in an electron-rich environment.

The spectrum is presented as a series of peaks on a scaled axis. The integration of each signal, which corresponds to the area under the peak, is proportional to the number of equivalent protons that are responsible for that signal. This allows for the determination of the relative ratio of different proton types within the molecule, providing essential clues for the structural assignment.

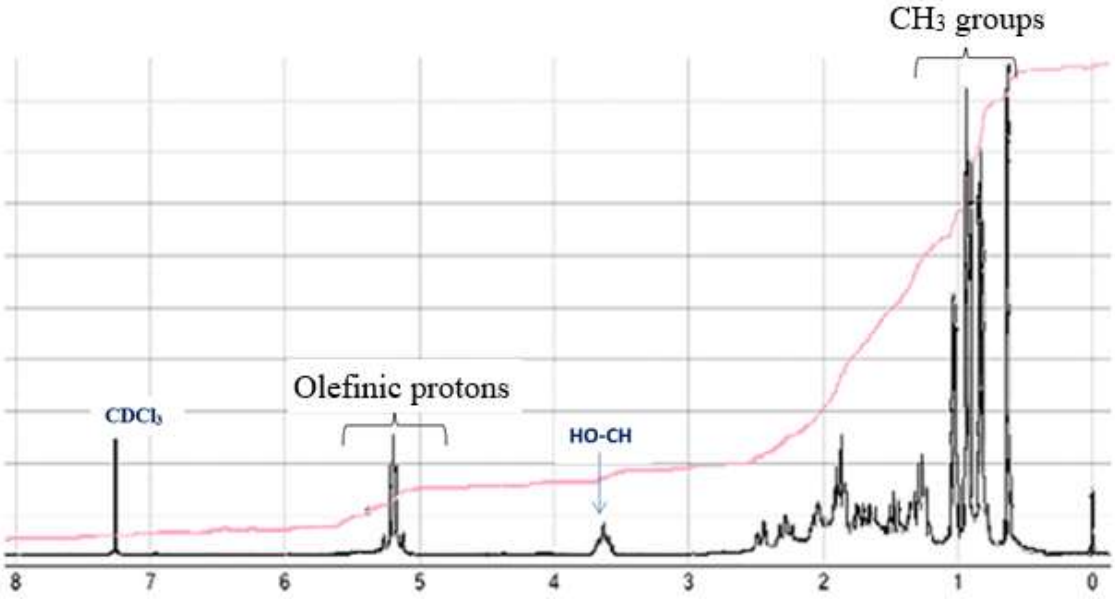



**Figure 3**. $^{1}$H NMR spectrum in $CDCl_3$ of the isolated compound

Analysis of the $^{1}$H NMR spectrum reveals spectral features consistent with a steroid framework. In the shielded region, a pair of prominent singlet signals at chemical shifts of **δ 0.6 ppm** and **δ 1.2 ppm** are characteristic of methyl ($CH_3$) groups. These are typically associated with the angular methyl groups at C-10 and C-13 of the steroid nucleus.

A one-proton signal appearing as a triplet at **δ 3.6 ppm** is highly indicative of a methine proton on a carbon bearing a hydroxyl group (**Harborne, 1998**). This chemical shift is a common signature for a secondary alcohol functionality in steroid molecules, such as a C-3 hydroxyl group. Further deshielding is observed in the olefinic region. A two-proton signal between **δ 5.2** and **δ 5.4 ppm** is attributable to an endocyclic olefinic moiety, such as a $CH_2$ =C− group within the steroid ring system (**Pavia et *al.*, 2015**). The presence and chemical shifts of these signals collectively provide strong spectroscopic evidence for the presence of a steroid backbone containing both a hydroxyl group and an olefinic unsaturation.

- **$^{13}$C NMR Spectra**

$^{13}$C NMR spectrum of the isolated compound reveals a total of 29 distinct signals, indicating the presence of 29 carbon atoms in the molecular structure. For a more detailed understanding of the carbon framework, **Distortionless Enhancement by Polarization Transfer (DEPT)** spectroscopy was performed. This technique is invaluable for classifying carbons based on the number of attached protons, differentiating between primary ($CH_3$), secondary ($CH_2$), tertiary (CH), and quaternary (C) carbons, which are typically not visible in DEPT spectra (**Pavia et *al.*, 2015**).

The **DEPT-90** experiment, with a pulse angle (θ) of 90°, selectively shows only the signals for methine (CH) carbons, which appear as positive peaks. This spectrum is particularly useful for identifying carbons that are part of a CH group.

The **DEPT-135** experiment, using a pulse angle of 135°, provides even more detailed information: it displays positive signals for both methyl ($CH_3$) and methine (CH) carbons, while methylene ($CH_2$) carbons are easily identified by their inverted (negative) signals (**Skoog et *al.*, 2017**). This strategic use of different DEPT experiments is crucial for a complete and accurate assignment of the carbon signals, thereby providing a clear map of the molecular skeleton.

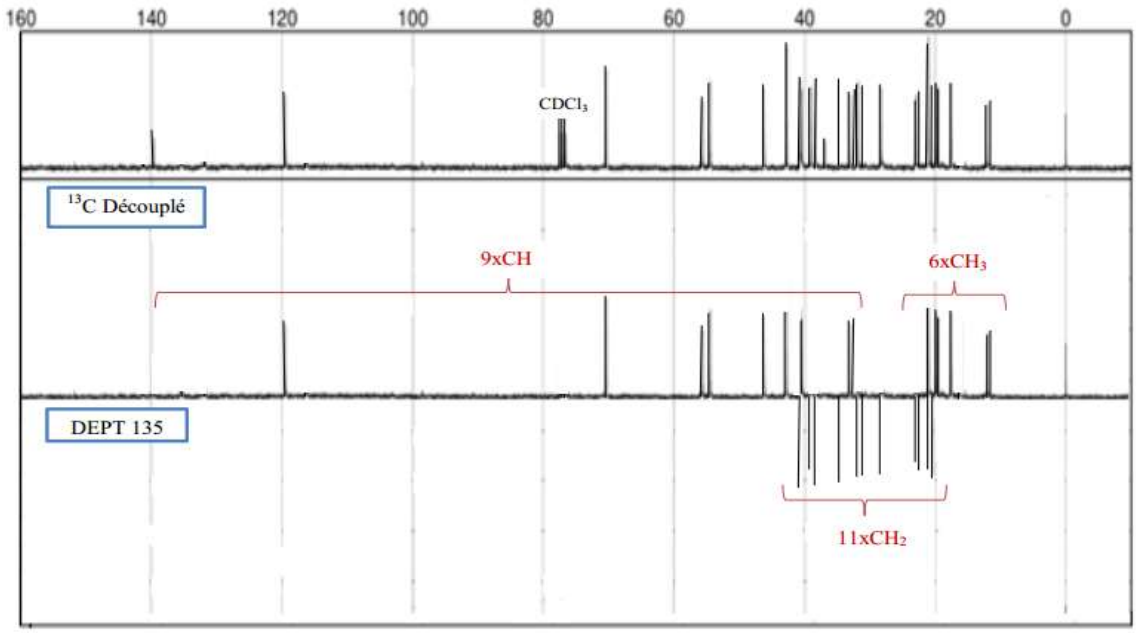



**Figure 4**. Results of $^{13}C$ NMR spectra, DEPT (135) of the isolated compound

Final analysis of the DEPT spectra provided critical information for determining the molecular formula and structure. The **DEPT-90** spectrum exhibited 9 signals, which correspond to nine methine (CH) groups. The **DEPT-135** spectrum confirmed this, showing 9 positive signals for CH groups, along with 6 positive signals for methyl ($CH_3$) groups and 11 negative signals for methylene ($CH_2$) groups.

A comparison of the DEPT spectra with the fully proton-decoupled $^{13}C$ NMR spectrum revealed three additional carbon signals that were not visible in the DEPT experiments. These are attributed to three quaternary carbons (Cq).

Based on this spectroscopic evidence, a proposed molecular formula can be derived. The presence of 6 $CH_3$ groups, 11 $CH_2$ groups, 9 CH groups, and 3 Cq carbons results in a total of 29 carbons and 50 hydrogens, leading to the molecular formula $C_{29}H_{50}O$. This formula has a molecular mass of 414 amu, which aligns perfectly with the molecular ion peak observed at m/z = 414 in the mass spectrum.

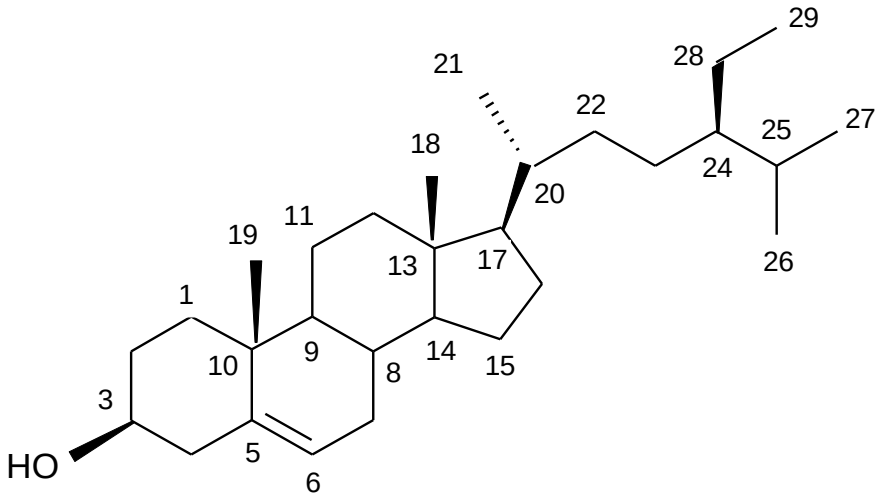
The chemical shifts for each carbon type further support the proposed steroid structure. The six methyl carbons were located at δ 12.1, 18.0, 19.7, 20.2, 21.4, and 29.8 ppm. The eleven methylene carbons appeared at δ 21.0, 21.4, 23.1, 24.3, 24.8, 28.3, 31.8, 36.5, 37.3, 42.8, and 42.8 ppm. The nine methine carbons were assigned to signals at δ 29.1, 31.9, 35.9, 45.6, 50.0, 55.9, 56.6, 73.4, and 121.7 ppm. Finally, the three quaternary carbons were found at δ 37.7, 42.8, and 140.0 ppm. The methine signal at **δ 73.4 ppm** is characteristic of a carbon bonded to a hydroxyl group, while the quaternary carbon at **δ 140.0 ppm** is indicative of an olefinic quaternary carbon.

The number of rings and degrees of unsaturation were calculated using the determined molecular formula ($C_{29}H_{50}O$). The calculation confirms five total degrees of unsaturation. The presence of two olefinic carbon signals in the $^{13}C$ NMR spectrum indicates one double bond, leaving four degrees of unsaturation attributable to four rings. This is consistent with a typical steroidal skeleton, which is composed of four fused rings.

**Table 2.** Procedure for obtaining the general formula

| Spectrum | Signal | Number of peaks | Formula |
|---|---|---|---|
| DEPT 90 | CH | 9 | $C_9H_9$ |
| DEPT 135 | $CH_2$ | 11 | $C_{11}H_{22}$ |
| | $CH_3$ | 6 | $C_6H_{18}$ |
| DEPT comparison with decoupled $^{13}C$ | Cq | 3 | $C_3$ |
| | OH | 1 | HO |
| General formula | | | $\mathbf{C_{29}H_{50}O}$ |

The analytical data, including the molecular mass of 414 amu and the presence of 29 carbon atoms, strongly supports a tetracyclic steroid structure. By comparing the collective spectroscopic data with established literature, the compound was definitively identified as **β-Sitosterol** . This identification is consistent with previous research on the phytochemistry of various plant species and confirms the presence of this well-known phytosterol (**Gomathi et *al.*, 2024**).




**IUPAC name :** 17-(5-Ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol.
**Figure 5.** Structure of β-Sitosterol

Based on the comprehensive analytical data, the elucidated structure is a **tetracyclic steroid** with a total of 29 carbon atoms, which is consistent with the determined molecular formula ($C_{29}H_{50}O$). Through a meticulous comparison of the compound's spectroscopic data, including Mass Spectrometry,

$^{1}H$ NMR, and $^{13}C$ NMR results, with established literature values, the structure was definitively identified as **β-Sitosterol** (**Gomathi et *al.*, 2024**).

***Tableau 1:*** *Comparaison des déplacements chimiques de composé isolé avec ceux de la littérature (**Gomathi et al., 2024**)*

| Number of carbon | Carbon type | Isolated compound (δ ppm) | Literature data (δ ppm) |
|---|---|---|---|
| C-1 | $CH_2$ | 37.3 | 37.3 |
| C-2 | $CH_2$ | 29.8 | 29.7 |

| | | | |
|---|---|---|---|
| C-3 | CH | 73.4 | 73.3 |
| C-4 | $CH_2$ | 42.8 | 42.9 |
| C-5 | Cq | 140.0 | 140.1 |
| C-6 | CH | 121.7 | 121.8 |
| C-7 | $CH_2$ | 28.3 | 28.3 |
| C-8 | CH | 31.9 | 31.8 |
| C-9 | CH | 50.0 | 50.1 |
| C-10 | Cq | 37.7 | 37.8 |
| C-11 | $CH_2$ | 21.4 | 21.2 |
| C-12 | $CH_2$ | 31.8 | 31.8 |
| C-13 | Cq | 42.8 | 42.3 |
| C-14 | CH | 56.6 | 56.5 |
| C-15 | $CH_2$ | 23.1 | 23.0 |
| C-16 | $CH_2$ | 24.8 | 24.7 |
| C-17 | CH | 55.9 | 55.8 |
| C-18 | $CH_3$ | 12.4 | 12.4 |
| C-19 | $CH_3$ | 21.4 | 21.5 |
| C-20 | CH | 35.9 | 36.0 |
| C-21 | $CH_3$ | 18.0 | 18.2 |
| C-22 | $CH_2$ | 36.5 | 36.5 |
| C-23 | $CH_2$ | 24.3 | 24.4 |
| C-24 | CH | 45.6 | 45.5 |
| C-25 | CH | 29.1 | 29.0 |
| C-26 | $CH_3$ | 19.7 | 19.7 |
| C-27 | $CH_3$ | 20.2 | 20.2 |
| C-28 | $CH_2$ | 21.0 | 21.1 |
| C-29 | $CH_3$ | 12.1 | 12.2 |

This table compares the 13C chemical shifts of the isolated product with those reported in the literature.

## IV. Conclusion

*Leptadenia madagascariensis* Decne is a plant species endemic to Madagascar, thriving in the warm, dry regions of the island due to favorable climatic conditions. This plant is known for its multifaceted value, which includes medicinal, ecological, strategic, aesthetic, economic, cosmetic, industrial, cultural, and nutritional uses.

This study focused on the phytochemical investigation of the aerial parts of this species to contribute to the national effort to conserve medicinal plants and promote local traditional medicine.

The plant sample was subjected to successive maceration using three solvents of increasing polarity: hexane, dichloromethane (DCM), and methanol. The resulting crude extracts yielded 3.93 mg, 3.14 mg, and 2.29 mg, respectively. Phytochemical screening tests on these extracts indicated a high content of polysaccharides, saponins, and steroids. These secondary metabolites are recognized for their significant therapeutic value.
Following these initial findings, the DCM extract was selected for further analysis due to its promising composition.

The DCM extract was first analyzed by thin-layer chromatography (TLC), which revealed four major components when eluted with a DCM/MeOH (99/01) solvent system. To isolate these components, the extract was then purified using column chromatography. This process yielded 102 fractions, with fractions 55 to 70 showing a single, pure compound upon TLC analysis. This fraction, designated F55-70, had a mass of 9.8 mg and was selected for structural determination.

The pure compound was characterized using mass spectrometry and one-dimensional nuclear magnetic resonance (NMR) spectroscopy, including $^{1}H$ NMR, $^{13}C$ NMR, and DEPT experiments. By comparing the obtained spectral data with established literature, the molecule was identified as β-Sitosterol, a compound belonging to the tetracyclic steroid family. This identification validates the presence of steroids, as initially indicated by the phytochemical screening.

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