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RESEARCH ARTICLE

Isolation, identification, and structure elucidation of Beta-sitosterol from Iraqi *Plantago major* using GC-MS, HPTLC, NMR, and FTIR

[version 1; peer review: awaiting peer review]

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Abstract

Background: *Plantago major*, a plant well recognized for its therapeutic features, has been widely adopted in several cultural instances and conventionally used due to its anti-inflammatory, antibacterial, and wound-healing characteristics. Recent research efforts have been focused on the identification and validation of the phytochemical elements of *P. major* in order to establish their association with their corresponding pharmacological effects. The main aim of this study is to precisely define, isolate, purify, and completely analyse the chemical composition of beta-sitosterol, a phytosterol obtained from the *Plantago major* plant native to Iraq.

Methods: In November 2021, *P. major* samples were procured from the Al-Salihiya Neighbourhood in Baghdad. These samples were afterward verified for authenticity by the Iraqi local Herbarium, located at the Al-Razi institute for alternative medicine. The desiccated botanical matter was subjected to a hexane-based defatting process inside a Soxhlet device, followed by gas chromatography/mass spectrometry (GC/MS) analysis in order to ascertain the presence of bioactive compounds. The evaluation of beta-sitosterol's presence was afterwards conducted via the use of conventional and preparative thin layer chromatography (TLC) methodologies and high-performance thin layer chromatography (HPTLC). The identification of the molecule was further validated by the use of Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) spectroscopy techniques.

Results: The findings revealed that Compound No. 5 had notable attributes that were consistent with those of Beta-Sitosterol. The

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provided data pertains to mass spectrometry (MS), especially the observation of a molecular ion peak at 414. This finding is consistent with the previously documented characteristics of beta-sitosterol. This finding provides further evidence supporting its classification as a prominent organic component originating from *P. major*.

Conclusions: We effectively determined beta-sitosterol as the primary bioactive component in the *P. major* samples collected. The compound's existence highlights the plant's longstanding therapeutic standing, ascribing it several advantageous pharmacological benefits.

Keywords

Plantago major, structure elucidation, phytochemical elements, Beta-Sitosterol, HPTLC

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Introduction

Plantago major, frequently referred to as broadleaf plantain, is a perennial herbaceous plant classified under the Plantaginaceae family, which includes other species of significant medicinal use, such as *Plantago lanceolata* and others.¹ The observed features include a basal rosette consisting of wide leaves with pronounced parallel veins. The leaves possess notable therapeutic properties and have been used in traditional medicine for a long time.² *P. major* has a vast distribution over many areas, including Europe, Asia, and North America, while demonstrating adaptability to a range of climates. This particular species is often seen inhabiting open fields, meadows, and roadside areas. The plant exhibits the growth of elongated spikes consisting of small, unremarkable blooms that often display hues of brown or green.³

The medicinal efficacy of *P. major* has been acknowledged in several cultural contexts.⁴ Historically, it has been conventionally used due to its anti-inflammatory, antibacterial, and wound-healing characteristics.⁵ This plant has a diverse array of bioactive constituents, including iridoid glycosides, flavonoids, tannins, and polysaccharides, which together serve its pharmacological properties.⁶

Numerous investigations have been conducted to examine the pharmacological properties of *P. major*, elucidating its potential therapeutic effects in the management of respiratory diseases, digestive dysfunctions, dermatological problems, and urinary tract infections.⁷ The plant has shown anti-inflammatory and antioxidant properties, which provide evidence to support its traditional use in the treatment of wounds and skin ailments.⁸

The field of pharmacognostical research encompasses a thorough analysis and description of botanical specimens with medicinal properties, aiming to ascertain their true nature, their efficiency, and determine their potential for therapeutic applications.^{9,10} These studies provide significant contributions to the understanding of several aspects of medicinal plants, including their botanical characteristics, macroscopic and microscopic features, chemical composition, and pharmacological properties.¹¹

Pharmacognostical investigations pertaining to *P. major* include the identification and analysis of its many botanical components, including leaves, stems, roots, and seeds, with the objective of ascertaining their therapeutic attributes.¹² The process of macroscopic inspection include the observation of the general characteristics, such as appearance, color, form, and texture, of the various components of the plant.¹³ Microscopic investigations include the analysis of slender plant material sections via the use of a microscope, with the aim of discerning distinct cellular structures, tissues, and other diagnostic attributes.^{14,15}

Previous research has shown that *P. major* contains several bioactive chemicals, including iridoid glycosides, flavonoids, tannins, phenolic compounds, and many others; these compounds are responsible for the plant's therapeutic effects.^{16,17} The primary objective of this work was to accurately ascertain, separate, refine, and comprehensively determine the chemical structure of the phytosterol known as beta-sitosterol, derived from the *P. major* plant indigenous to Iraq.

Methods

Ethical approval

Because of the research's reliance on plant analysis and chemical identification and the lack of direct contact with human participants or animal species, the Institutional Ethical Committee at College of Pharmacy, Mustansiriyah University, ruled this research free from ethical assessment.

Materials

Collection and preparation of plant material

The first step involved the acquisition of *P. major* specimens (whole plant) from the Al-Salihiya Neighborhood (Coordinates 33.323813, 44.390688) in Baghdad. The activity was conducted on November 2021. To confirm the accuracy of the plant species, it was necessary to verify them by consulting the Iraqi local Herbarium, which is located in the Al-Razi center for alternative medicine.

Shade drying

The plants were carefully cleansed by rinsing them with tap water in order to remove any accumulated dirt or dust particles. Next, it is recommended to carry out an additional rinsing step using distilled water in order to eliminate any residual pollutants that may still be present. The plants were subjected to a shade drying process for a duration of roughly 14 days. It is important to ensure that the drying space is devoid of direct sunshine and humidity at 28°C. After the plant material was dried, it was ground into a fine powder. The appropriate quantity needed for further testing was measured.

Table 1. Summary of chemicals and reagents.

Name of the chemical	Source
KOH	Fisher Scientific, USA
Ferric chloride	BDH Chemicals Ltd./England
Ethanol	Fluka/Switzerland
Methanol	Fluka/Switzerland
Ethyl acetate	HaymanKimia/UK
Acetic acid	Siuopharm Chemical Reagent Co.Ltd
Sulfuric acid	Hyperchem/China
Chloroform	Hyperchem/China
Hydrochloric acid	Alpha chemika/India
Aluminum chloride	Fluka/Switzerland
Acetic anhydride	Fluka/Switzerland
Sodium hydroxide	Hyperchem/China

Extract preparation

Following the drying process, the specimens were pulverized using a Willye-type mill (model TE650; Tecnal, Brazil). The resulting powder was carefully maintained in a light- and moisture-protected environment at a temperature of 28°C until it was ready for use. The extraction process included the use of a Soxhlet device, whereby 100 grams of powdered leaves were combined with 1 liter of n-hexane. The solvent underwent evaporation at a speed of 75 revolutions per minute at a temperature of 64.4 °C using an HB10 rotary-evaporator manufactured by IKA Works, located in Wilmington, NC, USA. The substance obtained during the process of solvent evaporation is referred to as the extract.¹⁸ A summary of chemical and reagent used in this study are included below in [Table 1](#).

Identification of beta-sitosterol from *Plantago major* by gas chromatography-mass spectrometry (GC-MS) analysis

To conduct the GC/MS test, it is recommended to use a volume of 1 µL from the hexane extract Helium gas as the mobile phase, maintaining a constant flow rate of 1.3 mL per minute. It is recommended to ensure that the temperature of the injection port is maintained at 250°C. The starting column temperature was set at 55°C, and it was gradually increased to 270°C over a duration of 5 minutes.^{16,19,20}

Isolation and purification of beta-sitosterol by thin-layer chromatography (TLC)

A thorough examination of the hexane fraction was conducted using both conventional and preparative thin-layer chromatography (TLC) techniques. The purpose of this procedure is to achieve the isolation and purification of the phytosterol Beta-sitosterol, followed by the subsequent elucidation of its structure. Prepare a standardized solution of Beta-sitosterol and conduct a comparative analysis with the hexane fraction extract using thin-layer chromatography plates. The Vanillin-Sulfuric acid may be used as a spraying reagent.

To perform thin-layer chromatography, it is recommended to use a mobile phase composed of Toluene, Ethyl acetate, and Glacial acetic acid in a proportion of 8:2:0.2.²¹ Upon the identification of Beta-sitosterol by thin-layer chromatography, the subsequent step involves the isolation of the chemical using preparative TLC.²² Precisely scratch off the band containing Beta-sitosterol from the thin-layer chromatography plate. The Beta-sitosterol may be isolated from the silica by using an acetone wash. Verification of the structural integrity and level of purity of the separated Beta-sitosterol by the use of Fourier transform infrared (FTIR) and Nuclear Magnetic Resonance (NMR) spectroscopic techniques.^{23,24}

Isolation and purification of beta-sitosterol by high-performance thin layer chromatography (HPTLC)

The optimization of the technique was conducted using stationary phase pre-coated silica gel 60 F254 glass sheets of 10 × 10 cm. The mobile phase used consisted of a mixture of anhydrous formic acid, acetone, methanol, and ethyl acetate in a volumetric ratio of 4:20:20:30. The reference and test solutions were seen as bands with a width of 5 mm, and the injection volume was established at 8 µL. The research was conducted inside a glass container measuring 20 × 20 cm, which consisted of twin troughs. The chromatographic chamber was allowed to reach saturation for a duration of about 30 minutes, while the migration distance permitted was set at 80 millimeters. The analysis of hederacoside C was conducted by subjecting it to a solution of 20% sulfuric acid in ethanol, followed by heating the plate at a temperature of 105°C and observing it under natural light conditions.^{25,26}

Table 2. highest-concentration phytochemicals in GC/MS of Hexane extract.

No.	Compound	RT (min)	Area	Area %
1	Benzene, 1-(1,5-Dimethy-4-Hexenyl)-4-Methyl-	52	26638158	7.38
2	9,12,15-Octadecatrienoic Acid	54.758	36242629	10.04
3	Naphthalene, 1,2,3,5,6,7,8,8a-Octahydro-1,8a-Dimethyl-7-(1-Methylethenyl)-, [1S(1a',7a',8aa')]]	56.147	51832930	14.36
4	Cis-13-Octadecenoic Acid, Methyl Ester	60.117	13257857	3.67
5	(3 β)-Stigmast-5-En-3-Ol	60.325	43440569	12.04
6	(3R,5E,9R)-3,9-Dimethyl-2,4,6,8-Nonatetraen-1-One	60.519	20335534	5.63
7	Lup-20(29)-En-3 β -Ol	60.657	20806533	5.76
8	(1S,2R,5R)-2-(4-Methyl-1-Cyclohex-3-Enyl) Propan-2-Ol	61.118	14342805	3.97
9	(3 β)-3-Hydroxyurs-12-En-28-Oic Acid	61.503	12984925	3.6
10	Sulfurous Acid, Cyclohexylmethyl Hexadecyl Ester	61.669	101197191	28.04
11	1-Aziridine Decanoic Acid, 2,2-Dimethyl-. Iota.- Oxo-, Ethyl Ester	62.371	19862270	5.5

RT: Retention time.

Results

The most prominent chemicals detected in the provided extract are presented in Table 2. Component number 5 was recognized as beta-sitosterol based on the mass spectrometry results of (refer to Figures 1 and 2), which included comparing the component with databases available in the GC/MS instrument.²⁷

Isolation and purification of beta-sitosterol from the hexane extract

The thin layer chromatography method was used to analyse the presence of beta sitosterol in the plant. A beta-sitosterol reference standard was used for comparison. The obtained findings, shown in Table 3, provided further evidence for the existence of beta-sitosterol in the plant.

Based on the aforementioned TLC findings which demonstrated the existence of beta-sitosterol, the extract underwent further purification procedures using preparative TLC to isolate the suggested band. Subsequently, the compound was further purified by preparative HPTLC with the aim of achieving a higher degree of compound purity as illustrated in Figure 3.

Structure elucidation for the proposed beta-sitosterol

Subsequently, the purified compound underwent examination by Fourier transform infrared spectroscopy (FTIR) Figure 4 and its interpretation in Table 4, and nuclear magnetic resonance (NMR) techniques to validate the identification of the isolated chemical, as seen in Figure 5 and its interpretation in Table 5.

Discussion

Upon careful analysis of the wide range of products obtained via the investigation process, it is imperative to acknowledge the profound importance of compound 5, as shown in Table 2. This particular molecule, due to its unique characteristics and properties, warrants significant consideration within the spectrum of elements now being investigated. The compound's identification as beta-sitosterol may be inferred from the MS data, as seen in Figure 2. The validity of this idea is supported by the presence of a molecular ion peak at 414, which aligns with the well-documented MS properties of beta-sitosterol.²⁸

The origin of beta-sitosterol is of special interest due to its main derivation from the *P. major* plant.²⁹ The production of beta-sitosterol in this plant is not a random occurrence, but rather an intrinsic characteristic of its chemical composition.³⁰ *P. major* has been the focus of various investigations and observations within the fields of herbal medicine and botany.³¹ Based on extensive research, it has been shown that the pharmacological advantages and impacts attributed to *P. major* are inherently connected to beta-sitosterol.^{32,33} The bioactive ingredient not only contributes to the medical capabilities of the plant but also plays a crucial part in characterizing them; therefore, gaining knowledge about the extraction process, features, and advantages of *Plantago major* may provide significant insights into the medicinal potential of this plant.³⁴

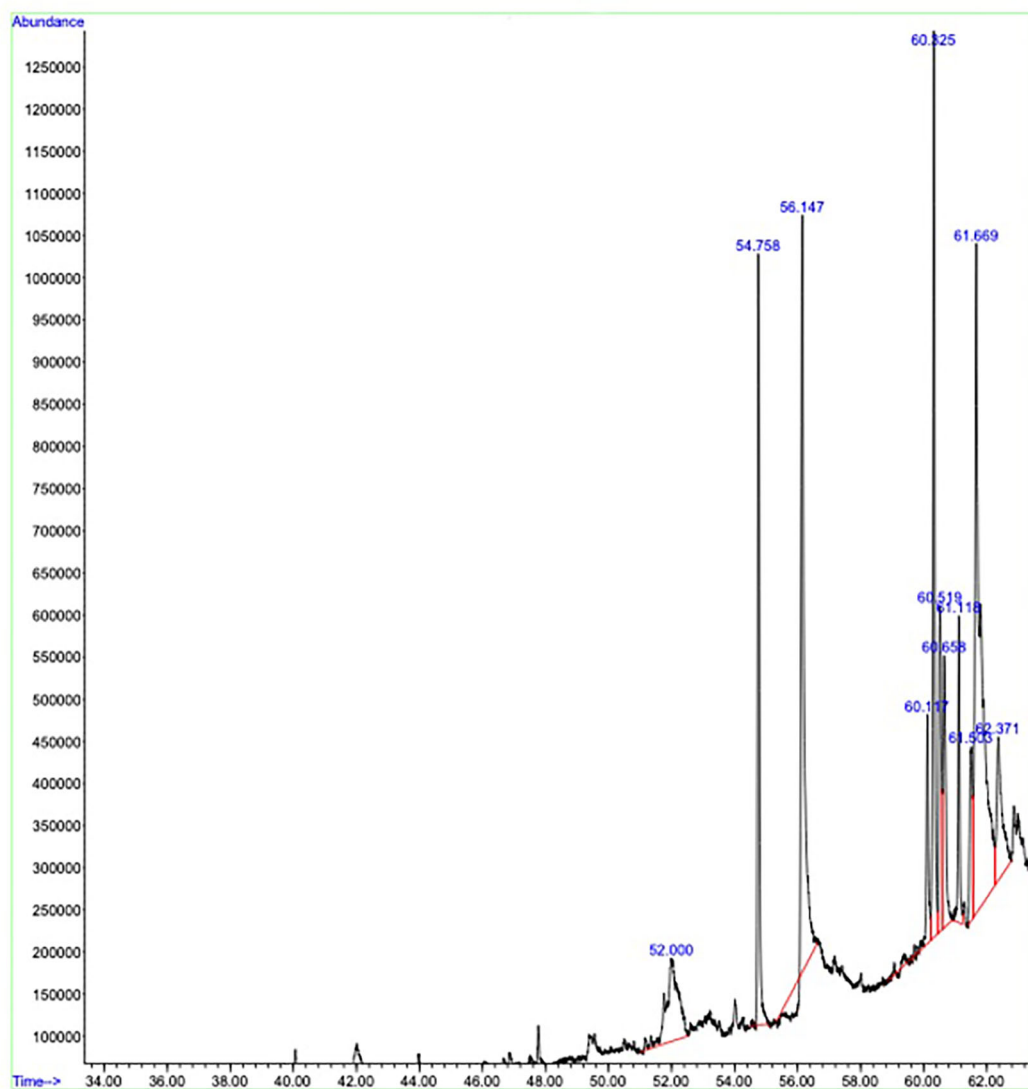


Figure 1. GC/MS chart of the hexane extract of the plant.

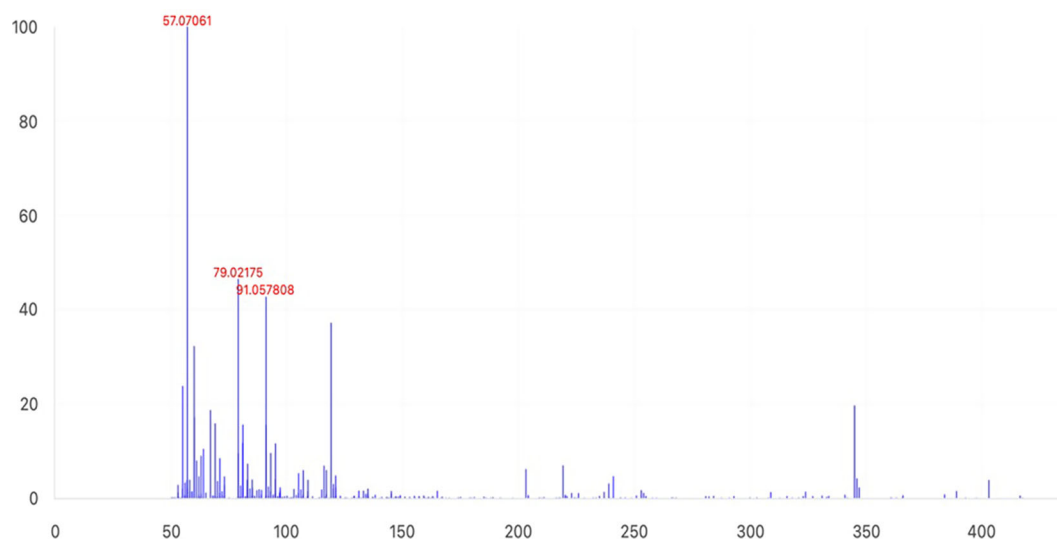


Figure 2. Mass spectroscopy chart of the proposed Beta-Sitosterol.

Table 3. Chromatography results for beta-sitosterol isolation.

Solvent system	R _f value of extracted beta sitosterol	R _f of reference standard
Ethyl acetate: Glacial acetic acid (8:2:0.2)	0.45	0.44

R_f: retention factor.

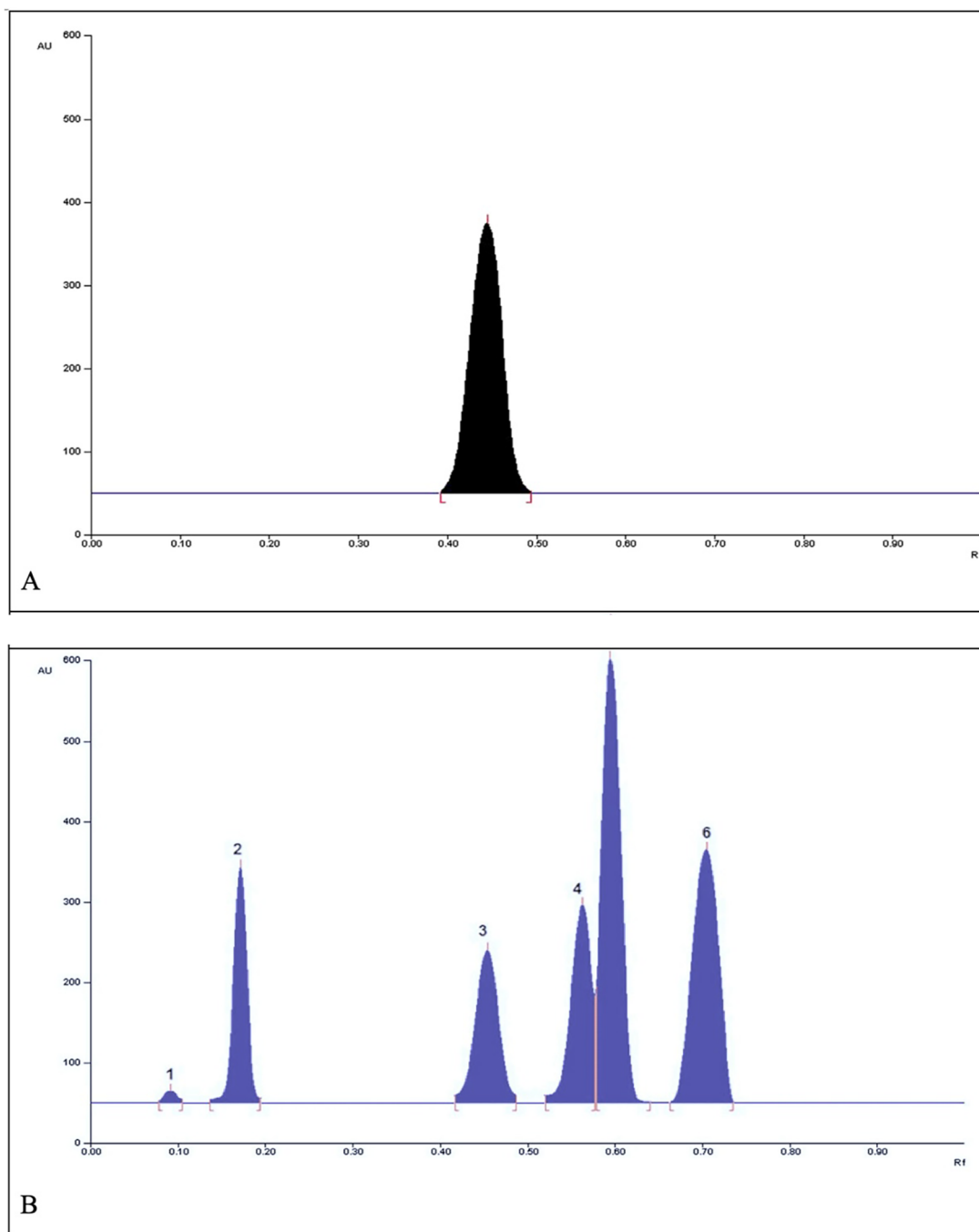


Figure 3. HPTLC chromatogram of beta-sitosterol, A: Beta-sitosterol standard, B: Hexane fraction in which beta-sitosterol is peak no 3.

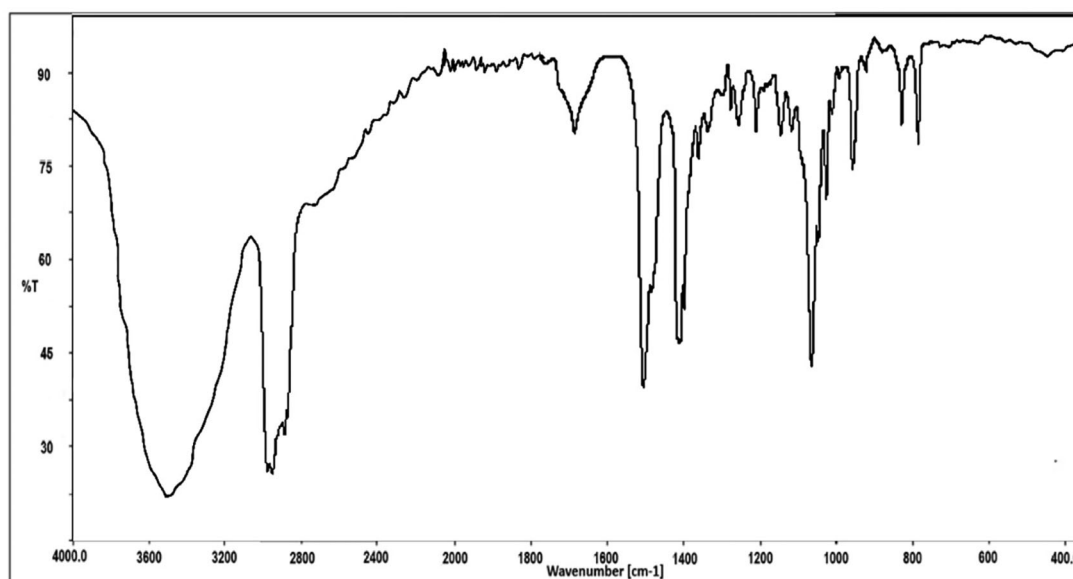
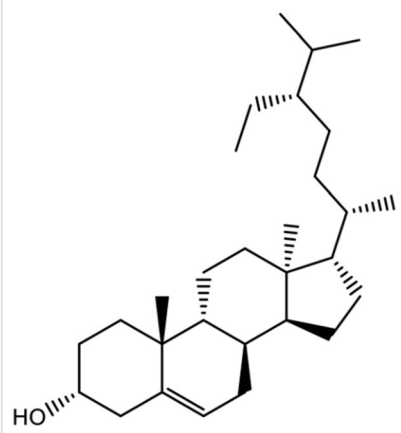


Figure 4. FTIR chart of the beta-sitosterol.

Table 4. FTIR results interpretation of beta-sitosterol.

Compounds	Bands (cm ⁻¹)	Interpretation
Beta-sitosterol 	3583	Stretching symmetric of hydroxy group
	1492	C=C Stretching of alkene

In this study the identification of the derived molecule was achieved by the integrated use of Fourier Transform Infrared Spectroscopy and Nuclear Magnetic Resonance analytical techniques. The findings obtained from this research exhibit a remarkable congruence with previous scholarly studies. Numerous investigations have consistently shown the presence of this particular phytochemical inside the plant. It is noteworthy to mention that a substantial proportion of the plant's recognized pharmacological advantages are often attributed to the existence of this specific chemical.^{35,36}

The use of hexane inside the Soxhlet apparatus not only eased this process but also contributed to the later discovery of bioactive compounds.³⁷

A notable aspect of this research was the successful identification of Compound 5 as beta-sitosterol. The importance of this cannot be underestimated. Beta-sitosterol, a sterol ester derived from plants, is present in plant cells and membranes and has been recognized for its potential physiological advantages,³⁸ such as its anti-inflammatory and cholesterol-reducing capabilities.³⁹

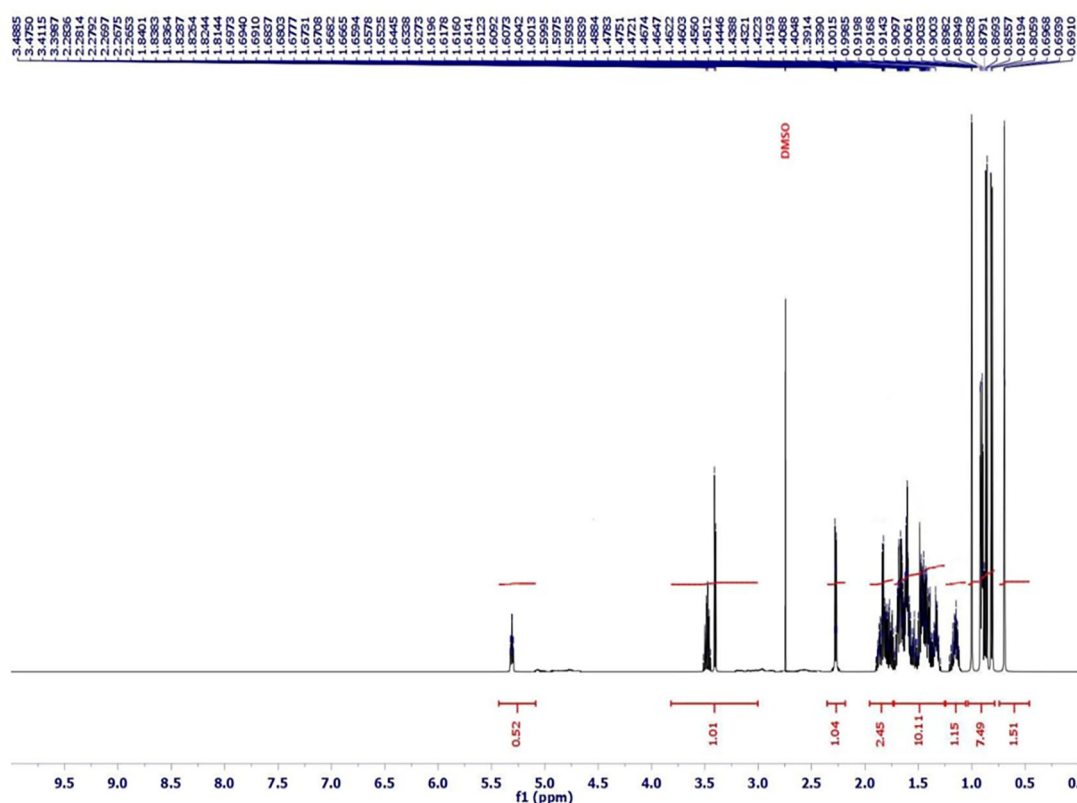


Figure 5. NMR chart of the beta-sitosterol.

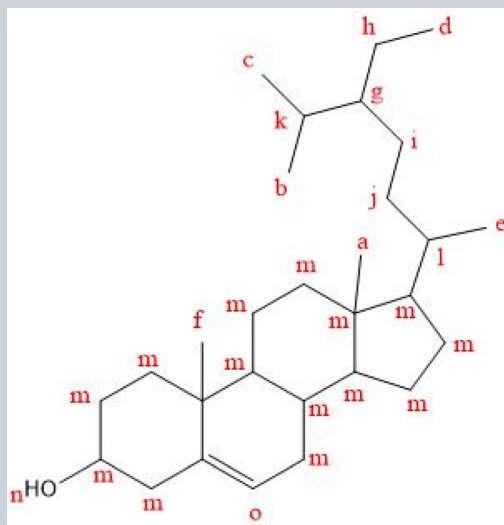
The use of TLC techniques provides a dependable means of verifying the existence of certain chemicals within a mixture. However, the incorporation of FTIR and NMR spectroscopy has enhanced the robustness and credibility of our results. Both of these methodologies, which are based on the examination of the molecular and atomic composition of substances, offered a comprehensive examination that was consistent with the current body of literature.⁴⁰

Moreover, the alignment of our results with prior research that demonstrates the existence of this phytochemical in this plant completes the cycle by validating its historic use. The observed link not only provides support for the validity of our research but also underscores the significance of beta-sitosterol in the pharmacological repertoire of *P. major*.³⁰

The presence of beta-sitosterol in the samples of *P. major* aligns with the longstanding assumption of the plant's therapeutic properties.⁴¹ As the investigation in this particular domain progresses, it becomes more essential to use a blend of conventional information and sophisticated methodologies to effectively exploit the medicinal capabilities of these botanical species.^{16,42}

Limitation of the study

The findings of this study provide insights into the detection of beta-sitosterol in *P. major* sourced from the Al-Salihiya Neighborhood in Baghdad, Iraq. However, it is crucial to recognize the potential variations in the phytochemical composition of samples obtained from diverse geographical areas or collected during different seasons. The quantity and profile of bioactive chemicals in plants may be substantially affected by environmental variables such as soil quality, rainfall, and temperature. Hence, it is important to acknowledge that the generalization of our results to include all *Plantago major* species may not adequately consider potential variances that may arise due to regional or seasonal factors.

Table 5. NMR interpretation of beta-sitosterol.

Group	Chemical shift ppm	NO. of H	Interpretation
a.	0.69	3	CH ₃ of Aliphatic part
b.	0.81	3	CH ₃ of Aliphatic part
c.	0.86	3	CH ₃ of Aliphatic part
d.	0.89	3	CH ₃ of Aliphatic part
e.	0.91	3	CH ₃ of Aliphatic part
f.	1.0	3	CH ₃ of Aliphatic part
g.	1.14	1	CH of Aliphatic part
h.	1.16	2	CH ₂ of Aliphatic part
i.	1.39	2	CH ₂ of Aliphatic part
j.	1.46	2	CH ₂ of Aliphatic part
k.	1.47	1	CH of Aliphatic part
l.	1.67	1	CH of Aliphatic part
m.	1.68-2.28	21	Multiplet of Cyclopentaphenanthrene
n.	3.41	1	Singlet of OH
o.	5.32	1	Triplet of alkene

Parts per million: ppm.

Conclusions

This study provides clear evidence of the existence of beta-sitosterol, a phytosterol compound that has great importance in the field of phytochemistry owing to its extensively studied medicinal properties. The findings of this study have significant significance for the area of natural product research, shedding insight on the possible medicinal uses of beta-sitosterol. As one of the most significant bioactive chemicals present in plants, its pharmacological activities have been widely investigated and recognized within the scientific community. The confirmed presence of beta-sitosterol highlights the significance of investigating its many medicinal capabilities, hence promoting a deeper comprehension of its methods of operation and prospective use in the creation of drugs and treatment approaches. The extensive examination of the compound's pharmacological activity has the potential to provide new opportunities for the creation of innovative pharmaceutical medicines, eventually leading to breakthroughs in human health and well-being.

Data availability

Underlying data

Zenodo: Isolation, identification, and structure elucidation of Beta-sitosterol from Iraqi *Plantago major* using GC-MS, HPTLC, NMR, and FTIR <https://doi.org/10.5281/zenodo.8247888>.²⁷

This project contained the following underlying data:

- Data.xlsx (Isolation, identification, and structure elucidation of Beta-sitosterol from Iraqi *Plantago major* using GC-MS, HPTLC, NMR, and FTIR).

Extended data

Zenodo: Supplement to: Isolation, identification, and structure elucidation of Beta-sitosterol from Iraqi *Plantago major* using GC-MS, HPTLC, NMR, and FTIR <https://doi.org/10.5281/zenodo.8336056>.⁴³

This project contained the following underlying data:

- Supplementary Appendix. Docx (Supplement to: Isolation, identification, and structure elucidation of Beta-sitosterol from Iraqi *Plantago major* using GC-MS, HPTLC, NMR, and FTIR).

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](https://creativecommons.org/licenses/by/4.0/) (CC-BY).

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