PROSPECTS
IN PHARMACEUTICAL SCIENCES

Prospects in Pharmaceutical Sciences, 23(1), 89-101
https://prospects.wum.edu.pl/

Original Article

ISOLATION AND CHARACTERIZATION OF Ƃ-SITOSTEROL AND Ƃ-CITRAURIN FROM SAPONIFIED PAPRIKA OLEORESIN

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Received: 12.11.2024 / Accepted: 27.12.2024 / Published: 05.03.2025

ABSTRACT

Capsicum annuum L. fruits, renowned for their nutritional and medicinal value, were explored as a source of valuable compounds. A method involving saponification, extraction, and column chromatography was utilized to isolate two significant compounds, Ƃ-sitosterol and Ƃ-citraurin, from paprika oleoresin. These compounds, known for their distinct properties, were identified through nuclear magnetic resonance, mass spectrometry, and chromatographic techniques. The newly developed method proved to be efficient, with reduced elution times, increased yields, and higher compound purity. The current study emphasizes the importance of *Capsicum annuum L.* fruits as a source of bioactive compounds and offers a valuable contribution to natural product research, suggesting potential applications in medicine and functional foods.

KEYWORDS: *Capsicum annuum L.*, Isolation, Ƃ-sitosterol, Ƃ-citraurin, Saponification.

1. Introduction

Phytochemicals are natural substances that are inherent to the plant kingdom. Natural products, such as plant extracts or standardized extracts, present unparalleled opportunities for new drug discovery due to their vast chemical diversity. Active constituents found in plants encompass a range of therapeutic effects on the human body. The World Health Organization (WHO) states that over 80% of the global population depends on traditional medicine to address their healthcare requirements [1-3]. Phytochemical screening of edible plants has gained prominence worldwide [1,2].

Capsicum annuum Linn., a member of the Solanaceae family, is an invaluable medicinal herb cultivated worldwide for fresh, dried, and processed products. This versatile fruit finds applications in various indigenous systems, including Siddha, Ayurveda, Unani, and Allopathy, for the treatment of various ailments. *Capsicum annum* L. contains capsaicin, capsaicinoids, and carotenoids. Alkaloids are compounds that are responsible for the pungency of chili peppers [4-6].

Bell pepper fruits, seeds, and leaves are rich in bioactive compounds, including carotenoids, flavonoids, phenols, pectic polysaccharides, and tocopherol, which exhibit antibacterial, antifungal, antioxidant, immunosuppressive, and immunostimulant properties. Some of these compounds, particularly flavonoids and carotenoids have been reported to possess potential antidiabetic, antitumoral, and neuroprotective effects, making them valuable candidates for functional food applications [7]. The vibrant color of paprika is attributed to its carotenoid content. Chilies are an abundant source of carotenoids, with approximately 25 different carotenoids identified in *Capsicum annuum L.* extracts, widely used in the food industry. The pigments responsible for its color include xanthophylls such as capsanthin, capsorubin, cryptoxanthin, lutein, zeaxanthin, and other carotenoids like α-carotene and Ƃ-carotene [5].

Capsicum oleoresin extracted from paprika is a complex mixture comprising essential oils, waxes, colored materials, and various capsaicinoids [8]. It is important to note that carotenoids cannot be synthesized within the human body and must be obtained through diet. Fruits

and vegetables are the primary dietary sources of carotenoids [9]. Carotenoids play a pivotal role in scavenging active oxygen species and possess provitamin activity, reducing oxidative stress in the human body. Their significant impact extends to preventing chronic diseases, including cardiovascular and cerebrovascular diseases, eye disorders, osteoporosis, and cancer [10].

In addition to carotenoids, phytosterols found in vegetables are known to be hypocholesterolemic and potent antioxidants. Despite their presence, phytosterol content in vegetable extracts often goes unnoticed. However, they have gained importance with the growing recognition of phytosterols in human nutrition [11]. ß-sitosterol, a plant sterol structurally similar to cholesterol, is widely found in vegetable oils, cereals, nuts, fruits, and beans. It is renowned for its cholesterol-lowering effects, anti-inflammatory, and antioxidant properties. Other phytosterols, such as campesterol and stigmasterol, share similar health benefits but differ slightly in their chemical structure and abundance. On the other hand, ß-citraurin, a carotenoid responsible for the red-orange color in paprika, is one of many carotenoids in *Capsicum annuum L.*, beside capsanthin, capsorubin, and cryptoxanthin. Carotenoids like ß-citraurin are potent antioxidants that play a crucial role in scavenging free radicals and reducing oxidative stress, contributing to the prevention of chronic diseases such as cardiovascular diseases and cancer. Other carotenoids, such as lutein, zeaxanthin, and ß-carotene, also found in paprika and other plants, share these antioxidant properties and are essential for eye health and immune function. The unique cholesterol-lowering ability of ß-sitosterol and the vibrant color and antioxidant properties of ß-citraurin highlight their significance among similar compounds in paprika and other plants [12,13]. Chili also boasts a high content of α-tocopherol, a potent antioxidant. Therefore, the assessment protocol for tocopherol and phytosterols holds significant relevance [11]. ß-sitosterol is a phytosterol known for its anti-inflammatory, cholesterol-lowering, and antioxidant properties [14,15], while ß-citraurin is a carotenoid recognized for its antioxidant activity and potential health-promoting effects [16,17].

The primary objective of this research was to develop a highly efficient and cost-effective procedure for isolating key bioactive compounds from paprika oleoresin, specifically ß-sitosterol, a sterol, and ß-citraurin, a carotenoid. Additionally, this study aimed to characterize the isolated compounds through spectral analysis and quantify their concentrations in the hexane extract using high-performance liquid chromatography (HPLC). The research encompassed characterizing the isolated compounds through nuclear magnetic resonance (NMR) and mass spectroscopy techniques and quantifying their concentrations in the initial hexane extract using high-performance liquid chromatography (HPLC). This integrated approach entailed optimizing the isolation procedure, identifying key chemical markers, performing in-depth structural characterization of the isolated compounds, and quantifying their abundance. By achieving these objectives, our research has advanced the understanding of sterols derived from paprika oleoresin while exploring their potential applications.

2. Materials and Methods

All solvents used in this study were distilled before utilization. For the isolation of ß-sitosterol and ß-citraurin, we utilized a range of solvents, including ethyl acetate, hexane, dichloromethane (DCM), acetone, methanol. Additionally, a solution of vanillin in sulfuric acid was used as a reagent for characterization.

2.1. Materials

The paprika oleoresin utilized for extraction and isolation was supplied by Sinopaprika Co. Ltd., Qingdao, China. Column chromatography was conducted using silica gel with a particle size ranging from 100-200 mesh supplied by Finar Chemicals, Gujarat, India. Thin-layer chromatography (TLC) was carried out using precoated silica gel 60 F254 plates supplied by Merck Ltd., New Jersey, USA. These TLC plates were derivatized using a vanillin-sulfuric acid system and supplied by Sigma-Aldrich, Karnataka, India.

2.2. Instruments

Chromatographic purity was evaluated using HPLC on an Agilent 1260 Infinity II HPLC system (Agilent Technologies, USA). The chromatographic separation was achieved on a C30 reverse-phase column (5 µm, 4.6 × 150 mm). The mobile phase consisted of ethyl acetate and hexane (75:25, v/v) with a flow rate of 1.0 mL/min. The detection wavelength was set at 450 nm using a photodiode array detector. The injection volume was 20 µL, and the total run time was 30 minutes. The column temperature was maintained at 30°C.

^{1}H NMR spectra were recorded on a JEOL JNM-ECZ500R at 500 MHz, and ^{13}C NMR spectra were recorded at 125 MHz, using $CDCl_3$ as the solvent. The sample concentration was approximately 10 mg/mL, and the analysis was conducted at room temperature (about 298 K). Chemical shifts were reported in parts per million (ppm) relative to tetramethylsilane (TMS) as the internal standard.

Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) analysis was conducted using a Waters Acquity ultra performance liquid chromatography-mass spectometry (ULPC-MS) system (Waters Corporation, Milford, USA) equipped with an electrospray ionization ion source. The chromatographic separation was achieved on a BEH C18 column (2.1 × 50 mm, 1.7 µm particle size) at a flow rate of 0.3 mL/min. The mobile phase was a gradient of acetonitrile and water, starting at 20% acetonitrile and reaching 80% acetonitrile over 8 minutes. The total run time was 10 minutes. Mass spectra were recorded in the positive ion mode with a capillary voltage of 3.5 kV. The mass range was set from 100 to 1000 m/z, and the cone voltage was 30 V with the desolvation temperature set at 400°C.

TLC was performed using a CAMAG TLC chamber (Anchrom Enterprises, Maharashtra, India) with silica gel 60 F254 plates (Merck, Germany). Various solvent systems were used to develop the plates. After spotting the sample, the plates were developed to a distance of 8 cm and visualized under ultraviolet light (450 nm).

2.3. Preparation of Reagents

The preparation of the vanillin-sulfuric acid reagent involved two steps: (i) Solution A: we accurately weighed 1 gram of vanillin and dissolved it in 200 mL of methanol. The solution was stirred for 5 minutes until complete dissolution. (ii) Solution B: we added 20 mL of sulfuric acid dropwise to 180 mL of water and sonicated the mixture for 5 minutes to ensure proper mixing.

Subsequently, both Solution A and Solution B were combined, and the mixture was stirred for an additional 2 minutes to obtain the final vanillin-sulfuric acid reagent.

2.4. Procedures

2.4.1. Saponification of Paprika Oleoresin

The saponification of Paprika Oleoresin was performed as follows: initially, 100 g of oleoresin were placed into a round-bottom flask (RBF), and saponification was carried out using 100 mL of a 25% ethanolic potassium hydroxide solution. The mixture was stirred in an oil bath for 5 hours at a temperature of 70°C. Afterward, the saponified oleoresin, referred to as sap-mass, was allowed to cool to room temperature. To the sap-mass, 50 mL of ethanol and 100 mL of water were added sequentially. The reaction mixture was then stirred for an additional 30 minutes using a magnetic stirrer to quench the reaction. The reaction mixture was then stirred for an additional 30 minutes using a magnetic stirrer to quench the reaction. The unsaponifiable fraction was discarded without further processing.

2.4.2. Extraction of Sap-mass

In the extraction of the sap-mass obtained from the previous step (the saponified paprika oleoresin reaction mass), the following procedure was followed: the sap-mass was transferred to a beaker, and 300 mL of hexane was added to it. The mixture was stirred for 30 minutes to ensure the complete dissolution of all saponified paprika mass. Subsequently, the mixture was transferred to a separating funnel, vigorously shaken for 3-4 minutes, and allowed to stand for 15-20 minutes to separate the organic and aqueous layers.

The two layers were carefully separated, and this extraction process was repeated ten times, each time using fresh 300 mL of hexane for each cycle, hence a total of 3 liters of hexane was utilized. All the hexane fractions obtained from the extractions were combined. To concentrate the hexane fraction, the combined solution was evaporated using a rotary evaporator under reduced vacuum conditions at 40°C for 2-3 hours, maintaining a pressure of 250 millibars.

2.4.2.1. Solvent System Used for the TLC Optimization of Saponified Paprika Oleoresin Hexane Extract

Various solvent systems were tested to determine the optimal one for separating the constituents on the TLC plate, and the results are presented in Table 1. Among the various solvent systems investigated, it was found that the ethyl acetate:hexane mixture in a ratio of 25:75% (v/v) provided the best separation for the constituents of the saponified paprika oleoresin hexane extract. This solvent system not only yielded effective separation but also reduced the required separation time when compared to the commonly used acetone:hexane solvent system typically employed in the laboratory.

Table 1. The solvent system used for TLC optimization of hexane concentrate

Serial No.	Solvent system
1	Ethyl acetate:hexane (10:90% v/v)
2	Ethyl acetate:hexane (25:75% v/v)
3	Acetone:hexane (5:95% v/v)
4	Acetone:hexane (35:65% v/v)
5	Dichloromethane:hexane (15:85% v/v)
6	Dichloromethane:hexane (25:75% v/v)
7	Dichloromethane:hexane (45:55% v/v)
8	Ethylacetate:dichloromethane (5:95% v/v)

2.4.2.2. Column Chromatography of Hexane Concentrate

The column chromatography of the hexane concentrate obtained in "Extraction of Sap-mass", which was the hexane extract of saponified paprika oleoresin, followed a systematic procedure. Initially, the hexane concentrates, weighing 10 g, were dissolved in a minimal amount of hexane and adsorbed onto silica gel (100-200 mesh size) at a ratio of 1:3. Subsequently, the mixture underwent drying under a rotary evaporator.

The first column, prepared for the chromatographic process, was loaded with 200 g of silica gel (100-200 mesh size) using hexane as the solvent for dry packing. The hexane extract, previously adsorbed onto the silica gel material (40 g), was then introduced into the column. Elution was performed using an ethyl acetate:hexane gradient system, with the ethyl acetate concentration ranging from 1% to 40% (v/v). Finally, the column was washed with 100% ethyl acetate. Fractions of 100 mL each were collected, and combined fractions were subjected to distillation using a rotary evaporator. Figure 1 represents a schematic representation of overall column chromatography.

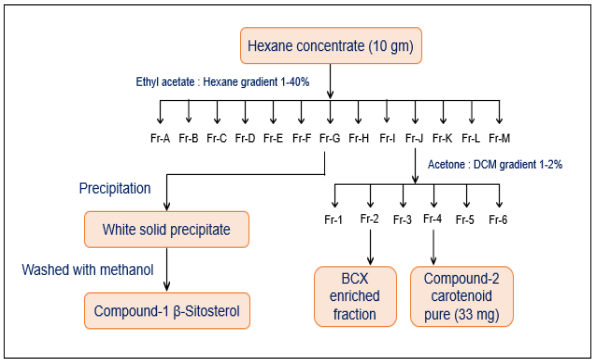

Fig. 1. Overall column chromatography

The obtained fractions were designated as follows: A (1170 mg), B (200 mg), C (560 mg), D (800 mg), E (300 mg), F (1.31 mg), G (870 g), H (200 mg), I (650 mg), J (200 mg), K (200 mg), L (223 mg), and M (column wash). Fig. 2 visually depicts these fractions. In this Figure, 2A shows column chromatography of hexane concentrate; 2B shows the TLC of isolated fractions from hexane concentrate, and 2C shows derivatized TLC of isolated fractions from hexane concentrate.

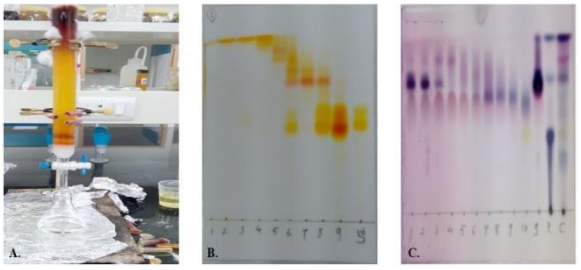

Fig. 2. (A: Column chromatography of hexane concentrate; B: TLC of fraction G; C: TLC of fraction G after derivatization with vanillin sulphuric acid).

All fractions underwent monitoring by TLC utilizing a mobile phase comprising ethyl acetate:hexane (25:75) on precoated silica gel GF254 plates. Subsequently, the TLC plates were derivatized with vanillin-sulfuric acid. Notably, fraction G exhibited a white precipitate. This precipitated fraction was washed with methanol and subjected to TLC analysis, revealing a distinct dark-pink-colored spot following derivatization with vanillin-sulfuric acid.

2.4.2.3. Recrystallization of Fraction

After the isolation of fraction G by column chromatography, which still contained some color impurities, fraction G was recrystallized using methanol. Fraction G was dissolved in methanol and shaken for 5 minutes, resulting in the formation of a white-colored precipitate. This precipitate was allowed to settle for 10 minutes. Subsequently, it was filtered off using Whatman filter paper and washed 3-4 times with methanol to remove any color impurities, finally, TLC was performed for recrystallized fraction (ß-sitosterol) which showed single dark violate spot after derivation with vanilline sulphuric acid as shown in Figure 3. Fraction G was then submitted for NMR spectroscopy.

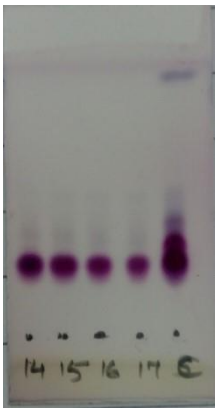

Fig. 3. TLC of ß-sitosterol derivatized with vanillin sulphuric acid

2.4.2.4. Solvent System Used for TLC Optimization of Fraction J

In the process of optimizing the TLC analysis for the fraction obtained, various solvent systems were evaluated, and the results are summarized in Table 2. Among these different systems, it was determined that the ethyl acetate:DCM (1:99) isocratic solvent system exhibited the most favorable performance for the isolation of fraction J. This solvent system allowed for the combination of fractions 7, 8, 9, and 10, effectively isolating fraction J. The application of this solvent system resulted in an impressive 88% chromatographic purity, as calculated by area analysis.

Table 2. Solvent system used for TLC optimization of fraction J

Serial No.	Solvent system
1	Dichloromethane:hexane (10:90 v/v)
2	Dichloromethane:hexane (50:50 v/v)
3	Acetone:hexane (5:95 v/v)
4	Ethyl acetate:dichloromethane (1:99v/v)

2.4.2.5. Column chromatography of fraction J

Fraction J, weighing 200 mg, was dissolved in the minimum required amount of DCM and subsequently adsorbed onto silica gel (100-200 mesh size) at a ratio of 1:5. The material was then dried under a rotary evaporator. For the initial column packing, 30 g of silica gel (100-200 mesh size) was employed, with DCM serving as the dry packing solvent.

The adsorbed material (40 g) was loaded onto the column and subjected to elution using acetone:DCM isocratic system (2% acetone:DCM). Following elution, the column was washed with 100% acetone. Fractions of 10 mL each were collected and subsequently distilled using a rotary evaporator, resulting in the isolation of fractions 1 to 10.

All fractions (1, 2, 3, 4, 5, 6, 7, 8, 9, 10) underwent monitoring via TLC employing a mobile phase comprising acetone:DCM (0.05:10) on precoated silica gel GF254 plates. The TLC plates were derivatized with vanillin-sulfuric acid and heated for 10 minutes on a hot plate. Among these fractions, fraction 4 displayed a distinct blue-colored single spot on the TLC plate after derivatization with vanillin-sulfuric acid reagent.

Fraction 4 was then submitted for chromatographic analysis and analyzed using a C30 column with an ethyl acetate and hexane gradient solvent system on an Agilent USA instrument. The calculated chromatographic area percentage revealed a chromatographic purity of 88%. Finally, fraction 4 was submitted for NMR and mass analysis.

Fig. 4 shows the column chromatographic separation of ß-citraurin (combined fractions 7, 8, 9, 10) and TLC showing a dark blue color spot due to ß-citraurin, separated by using an ethyl acetate:DCM (1:99) solvent system.

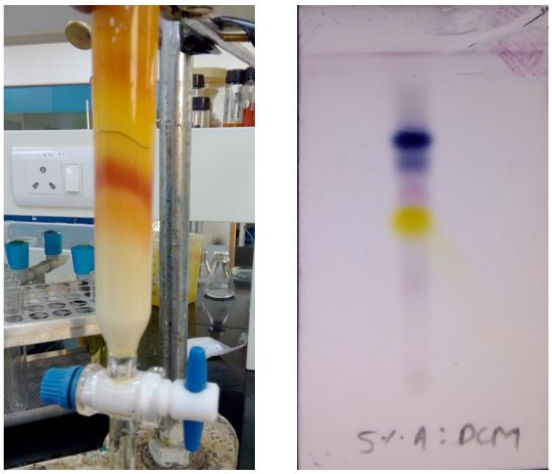

Figure 4. Column chromatography and TLC of ß-citraurin

Further TLC was monitored by using a 5% acetone and DCM solvent system.

3. Results and Discussions

Among the various solvent systems that were tested, it was determined that the ethyl acetate:hexane (25:75% v/v) combination proved to be the most suitable for the intended purpose. This solvent system exhibited the

highest compound purity and required less elution time compared to the laboratory's commonly used acetone:hexane (2:98% v/v) system for isolating desired constituents. The acetone:hexane solvent system was found to be less efficient, necessitating more time for the elution of fractions from the column. Following derivatization with vanillin-sulfuric acid, the ethyl acetate:hexane system consistently yielded clear separation of fractions on the TLC plates, as depicted in Figures 2B, 2C, and 3.

3.1. HPLC Analysis of Isolated Compound

Following the isolation of fraction G through the column chromatography procedure described in the methodology section, a percentage purity analysis of isolated ß-sitosterol was conducted using a C30 column. Elution was carried out with an ethyl acetate and hexane gradient solvent system on the Agilent USA HPLC System. The yields obtained from the isolated fractions are presented in Table 3, and the HPLC chromatogram of ß-sitosterol is shown in Figure A1 (Appendix).

Table 3. Details of physical characteristics and yield of isolated compounds

Serial No.	Sample	Fraction code	Color	Yield (g)	% Yield
1	ß-sitosterol	Fraction-G	White powder	0.300	15
2	ß-citraurin	Fraction-J	Red colored waxy material	0.200	22

3.2. Stability and Storage Precautions for ß-citraurin

Carotenoids are highly unstable and light-sensitive compounds. They are prone to degradation, especially at room temperature. To preserve their integrity, it is advisable to store these compounds at a temperature of 5°C.

3.3. Structure Elucidation of Isolated Compounds (ß-sitosterol)

The isolated fraction G, as discussed in the column chromatography of hexane concentrate and recrystallization of fraction sections, underwent structural characterization. ^{1}H NMR and ^{13}C NMR spectra were recorded in deuterated chloroform ($CDCl_3$) at the Department of Chemistry, Council of Scientific and Industrial Research-National Chemical Laboratory, Pune. From ^{1}H and ^{13}C NMR analysis, compound-1 was identified as ß-sitosterol (Fig. 5).

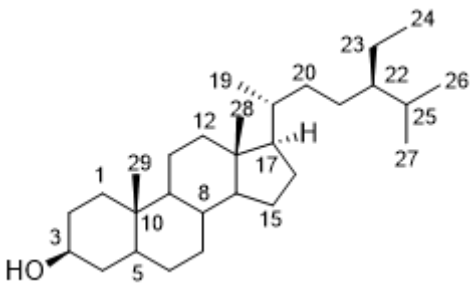

Fig. 5. Structure of ß-sitosterol

ß-sitosterol was isolated as a white powder with a yield of 300 mg. A pseudo-molecular peak (M+k) at 435.80 ($C_{29}H_{48}O$, cald: 413.53 g/mol) in the LC-ESI-MS allowed for the determination of its molecular formula as $C_{29}H_{48}O$, corresponding to six degrees of hydrogen deficiency. ^{1}H NMR and ^{13}C NMR (see Table A1; Appendix) indicated the presence of two tertiary methyl groups at δH 0.68 and 1.01, three secondary methyl groups at δH 0.83, 0.85, and 0.94, one primary methyl at δH 0.86, and one methine signal at δH 71.8 (as shown in Figures A2 and A3; Appendix). Distortionless Enhancement by Polarization Transfer (DEPT)-135 NMR revealed the presence of nine methylenes (as shown in Figure A4; Appendix). The ^{13}C NMR signals at δ 140.73, 121.70, 138.32, and 129.22 indicated the presence of one di-substituted and one tri-substituted double bond, suggesting the existence of a tetracyclic steroidal skeleton. The presence of a tetracyclic steroidal skeleton, as suggested by the ^{1}H and ^{13}C NMR data, in conjunction with the LC-ESI-MS and TLC analysis using the authenticated sample 4, was further confirmed by comparing the observed data with literature NMR data [18].

3.4. Structure Elucidation of Isolated Compounds (ß-citraurin)

ß-citraurin exhibits strong absorption in the range of 400–500 nm, corresponding to its conjugated polyene structure. This region is characteristic of carotenoids such as ß-carotene, α-carotene, lycopene, and ß-cryptoxanthin, and is responsible for their orange to red-coloration. In this study, the isolated compounds were characterized using mass spectrometry, ^{1}H NMR, and ^{13}C NMR spectroscopy [19].

ß-citraurin was isolated as a red-colored waxy material with a yield of 200 mg. A pseudo-molecular peak (M+k) at 433.10 ($C_{30}H_{40}O_2$, cald: 432.30) in the LC-ESI-MS allowed for the determination of its molecular formula as $C_{30}H_{40}O_2$. From the ^{1}H and ^{13}C NMR analysis, compound-1 was identified as ß-citraurin (see Fig. 6).

The ^{1}H and ^{13}C NMR data (refer to Table A2; Appendix) indicate the presence of a hydroxyl group at δH 4.8, two methyl groups attached to the cyclic ring at 37.11 (C-1), eight double bonds at 137.69 (C-6), 138.37 (C-8), and (C-9), and a deshielded proton signal at δH 12.70, suggesting the presence of an oxygen-containing functional group, possibly a hydroxyl or carboxyl moiety, at C-20. Fig. A5 provided in Appendix shows the ^{1}H NMR spectrum of ß-citraurin, and Figures A6-9 (Appendix) show several regions of the ^{13}C NMR spectrum of ß-citraurin.

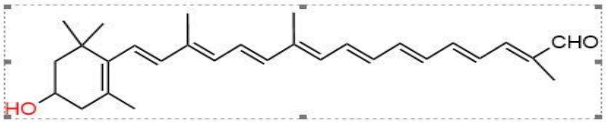

Fig. 6. Structure of ß-citraurin

3.5. Mass Spectrum in Text Format

The LC-ESI-MS analysis of ß-citraurin displayed a prominent pseudo-molecular ion peak at *m/z* 433.10 ([M+H]+), consistent with its molecular formula, $C_{30}H_{40}O_2$ (calculated molecular weight: 432.30 g/mol). The fragmentation pattern revealed a key fragment at *m/z* 417.10, corresponding to the loss of a hydroxyl group (-OH). Additionally, a sodium adduct peak was observed at *m/z* 455.30 ([M+Na]+), indicating the presence of polar functional groups, such as hydroxyl groups, that interact with metal ions during ionization.

ß-citraurin: LC-ESI-MS: m/z 433.10 [M + 1]+, 417.10 [M - OH]+, 455.30 [M + Na]+ ($C_{30}H_{40}O_2$, cald: 432.30); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 9.46 (H-8', singlet), 6.97-6.94 (H-10', doublet, J = 11 Hz), 6.77-6.65 (H-7, H-8, H-11', H-12', H-15', multiplet), 6.44-6.47 (H-11, doublet, J = 12 Hz), 6.36-6.40 (H-15, doublet, J = 15 Hz), 6.27-6.30 (H-12, doublet, J = 12 Hz), 6.14-6.19 (H-10, H-14, H-14', multiplet), 5.37 (3-OH), 4.01 (H-3, multiplet), 2.37 (H2-4, multiplet), 2.01 (H3-19, H3-20, singlet), 1.99 (H3-20', singlet), 1.91 (H3-19', singlet), 1.80 (H2-2, multiplet), 1.75 (H3-18, singlet), 1.08 (H3-16, H3-17, singlet). From the ^{1}H and ^{13}C analyses, DEPT analysis, and mass spectrum, the compound was identified as ß-citraurin. The molecular peak at (M+k) for the compound is 423.35, as shown in Fig. A10 (Appendix).

3.6. Comparison with Existing Literature

The choice to study the fruits of *Capsicum annuum L.* in this research is based on their well-documented nutritional value, medicinal uses, and strong antioxidant properties. Paprika oleoresin underwent saponification with sodium hydroxide, and hexane was selected as the extraction solvent due to its effectiveness in dissolving non-polar compounds. Non-polar solvents such as hexane, petroleum ether, or tetrahydrofuran are particularly efficient for extracting non-polar carotenes or esterified xanthophylls. The saponification method proved successful in removing unwanted lipids and esters, thereby facilitating the identification of the main carotenoid pigments in the natural extract [20].

In this study, we successfully isolated two compounds from plant sources: ß-sitosterol and ß-citraurin, both sourced from paprika oleoresin (*Capsicum annuum L.*). The yields for ß-sitosterol and ß-citraurin were 15% and 22%, respectively. ß-sitosterol is characterized by its white color, while ß-citraurin displays a red color.

The optimization of TLC for the initial hexane concentrate revealed that an ethyl acetate:hexane mixture in a 25:75% (v/v) ratio was the most effective solvent system for isolation. This choice not only provided efficient separation but also reduced the required elution time compared to other solvent systems.

The column chromatography of the hexane concentrate led to the isolation of 300 mg of ß-sitosterol. Subsequently, TLC optimization for the combined fractions 7, 8, 9, and 10 revealed that acetone:DCM (1:99) isocratic solvent system was the most favorable for the isolation of fraction J. Column chromatography of fraction J yielded 200 mg of ß-citraurin.

During analysis, fraction G exhibited a pseudo-molecular peak (M+k) at 435.80 ($C_{29}H_{48}O$, cald: 413.53 g/mol) in the LC-ESI-MS, allowing for the determination of its molecular formula as $C_{29}H_{48}O$, with six degrees of hydrogen deficiency. The ^{1}H NMR and ^{13}C NMR data indicated the presence of various methyl and methine groups. The ^{13}C NMR signals confirmed the existence of di-substituted and tri-substituted double bonds, suggesting a tetracyclic steroidal skeleton.

In the case of fraction J, a pseudo-molecular peak (M+k) at 433.10 ($C_{30}H_{40}O_2$, cald: 432.30 g/mol) was observed in the LC-ESI-MS, leading to the determination of its molecular formula as $C_{30}H_{40}O_2$. The ^{1}H and ^{13}C NMR data pointed towards the presence of a hydroxyl group, methyl groups attached to the cyclic ring, multiple double bonds, and an oxygen signal.

This developed method has proven to be superior compared to other reported methods, offering benefits such as reduced elution time, increased compound yield, and higher purity of the isolated compounds.

The isolation and characterization of ß-sitosterol and ß-citraurin from *Capsicum annuum L.* align with previous studies that have highlighted the presence of these compounds in various plant sources. For instance, ß-sitosterol is a well-known phytosterol with significant pharmacological properties, including anti-inflammatory, anticancer, and cholesterol-lowering effects [21]. Similarly, ß-citraurin, a carotenoid, has been recognized for its antioxidant properties and potential health benefits [22].

Our findings are consistent with the literature, which reports the presence of ß-sitosterol in many plant species and its role in reducing cholesterol levels and providing anti-inflammatory benefits [23,24]. The structural elucidation of ß-sitosterol through NMR and mass spectrometry in our study corroborates the data reported by Chaturvedula and Prakash (2012), further validating our results [18].

In the case of ß-citraurin, our study confirms its presence in *Capsicum annuum L.* and its structural characteristics as identified through NMR and mass spectrometry. The antioxidant properties of ß-citraurin, as reported in the literature, support its potential use in preventing oxidative stress-related diseases [23].

3.7. Potential application in industries

The bioactive properties of ß-sitosterol and ß-citraurin suggest their potential applications in various industries. ß-sitosterol is utilized in food products like dairy and vegetable oils to promote heart health. It is recognized for its ability to lower LDL cholesterol levels by reducing cholesterol absorption in the intestines [25]. In the pharmaceutical industry, ß-sitosterol's cholesterol-lowering and anti-inflammatory effects make it a valuable component in the development of treatments for cardiovascular diseases and inflammatory conditions. Additionally, its anticancer properties open avenues for its use in cancer therapy [13].

In the food industry, ß-citraurin's antioxidant properties and vibrant color make it an ideal natural food additive. It can be used to enhance the nutritional value and visual appeal of food products, while also providing health benefits by reducing oxidative stress. The use of natural colorants like ß-citraurin aligns with the growing consumer demand for clean-label products [26,27].

These applications highlight the significance of ß-sitosterol and ß-citraurin, not only as bioactive compounds but also as valuable ingredients in the pharmaceutical and food industries.

4. Conclusions

In this research, our focus was directed toward the *Capsicum annuum L.* fruits, renowned for their nutritional and medicinal value, as a prominent source of bioactive compounds. Our method, which involved a precisely executed process of saponification, extraction, and column chromatography, led to the successful isolation of

two significant compounds, ß-sitosterol and ß-citraurin, derived from paprika oleoresin. These compounds, distinguished by their unique physical properties, were effectively identified through the adept application of NMR, mass spectrometry, and chromatographic techniques. Our newly developed methodology has emerged as highly efficient, notable for its ability to reduce elution times, enhance compound yields, and ensure a higher degree of compound purity. This advancement contributes significantly to the domain of natural product isolation and characterization, offering promising prospects for further investigations into the medicinal, nutritional, and industrial applications of these compounds.

Future research should explore the in vivo effects of ß-sitosterol and ß-citraurin to better understand their pharmacokinetics and therapeutic potential. Investigating their bioavailability and metabolism in animal models and human subjects will provide valuable insights into their efficacy and safety. Additionally, the potential use of ß-citraurin as a natural food preservative due to its antioxidant properties warrants further study. This could lead to the development of new food preservation techniques that enhance shelf life while maintaining nutritional quality.

Moreover, the application of ß-sitosterol in functional foods and nutraceuticals could be expanded, given its cholesterol-lowering and anti-inflammatory effects. Exploring its incorporation into dietary supplements and fortified foods could offer new avenues for promoting cardiovascular health. Similarly, ß-citraurin's vibrant color and health benefits make it an ideal candidate for natural food colorants, aligning with the growing consumer demand for clean-label products.

Overall, the promising results of this study lay the groundwork for future research and development, aiming to harness the full potential of these bioactive compounds in various industries.

Appendix

Figures:

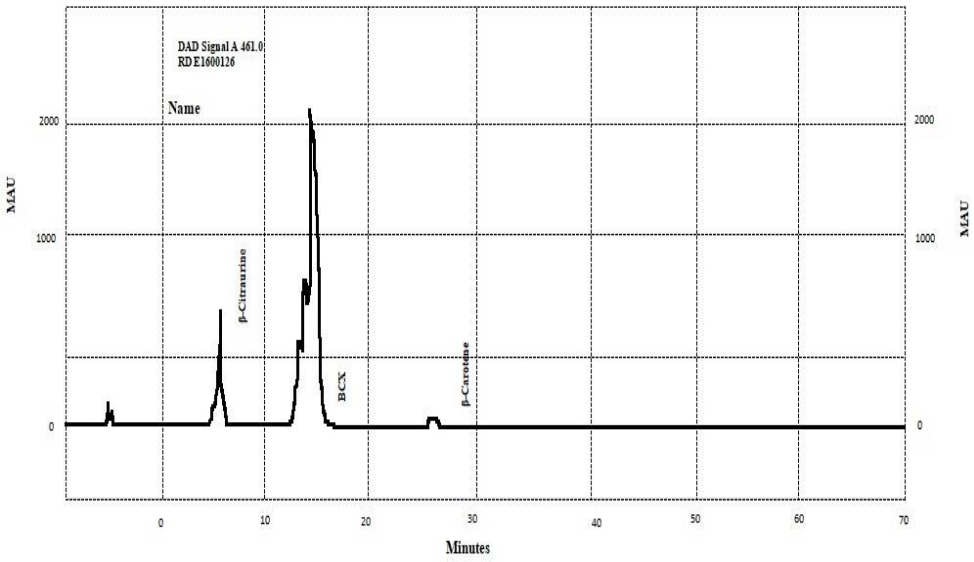

Fig. A1. HPLC Chromatogram of ß-citraurin

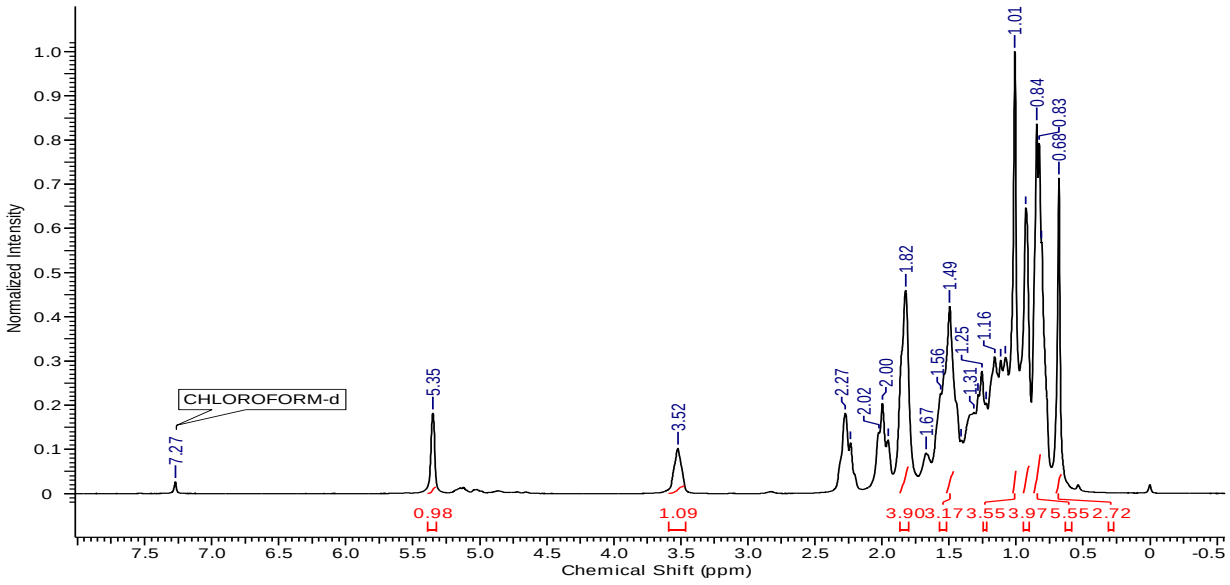

Fig. A2. ^{1}H NMR spectrum of ß-sitosterol

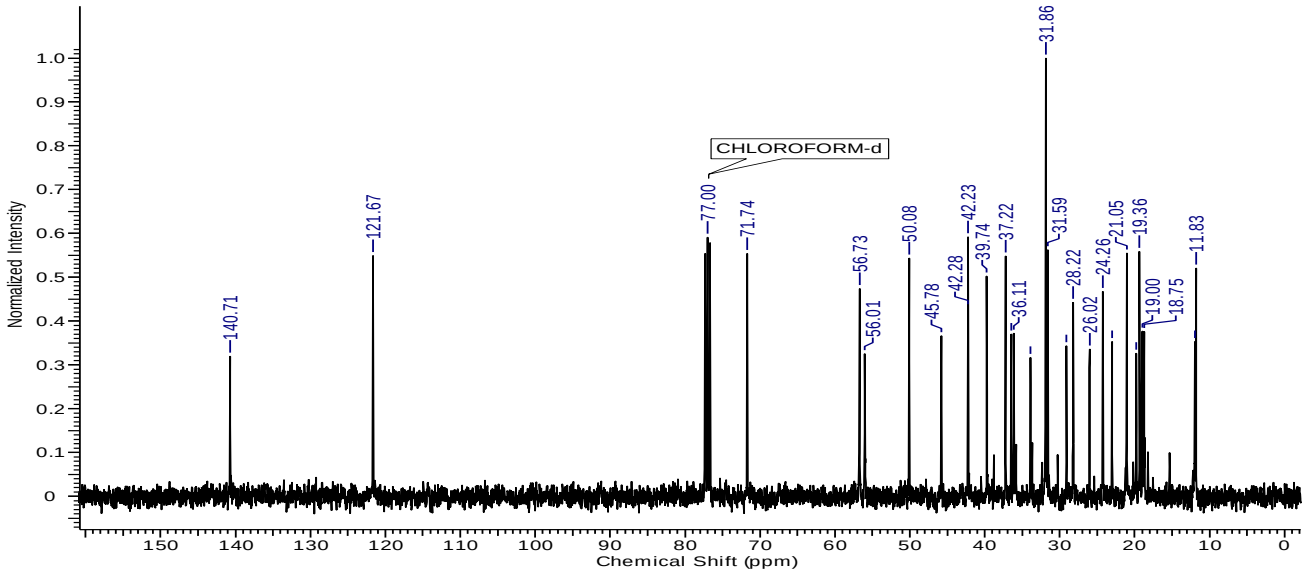

Fig. A3. ^{13}C NMR spectrum of ß-sitosterol

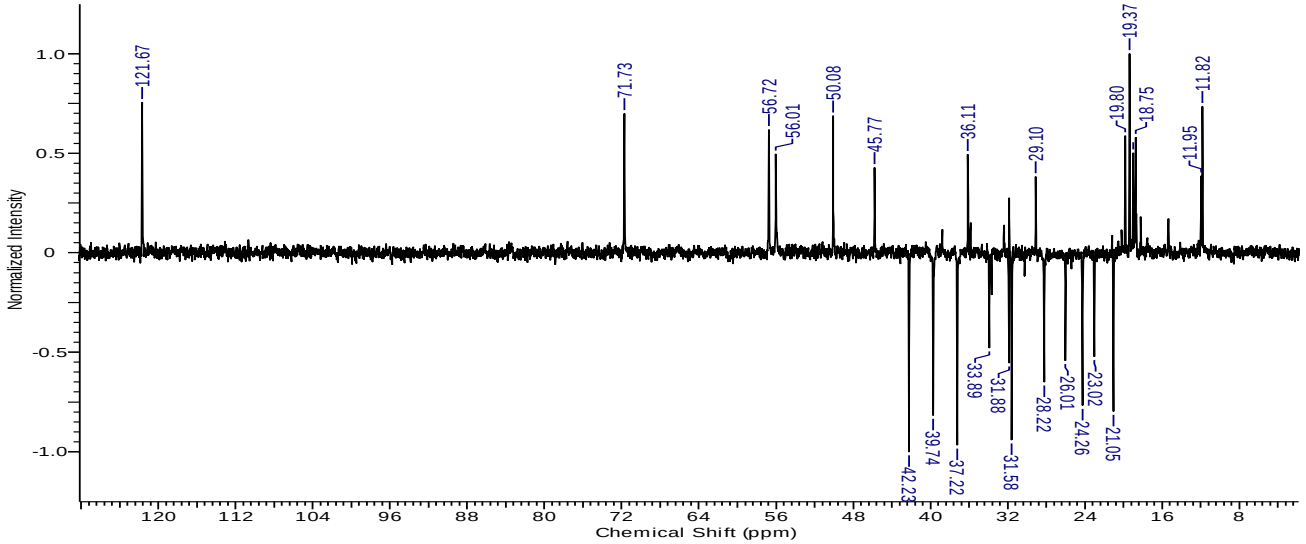

Fig. A4. DEPT-135 NMR spectrum of ß-sitosterol

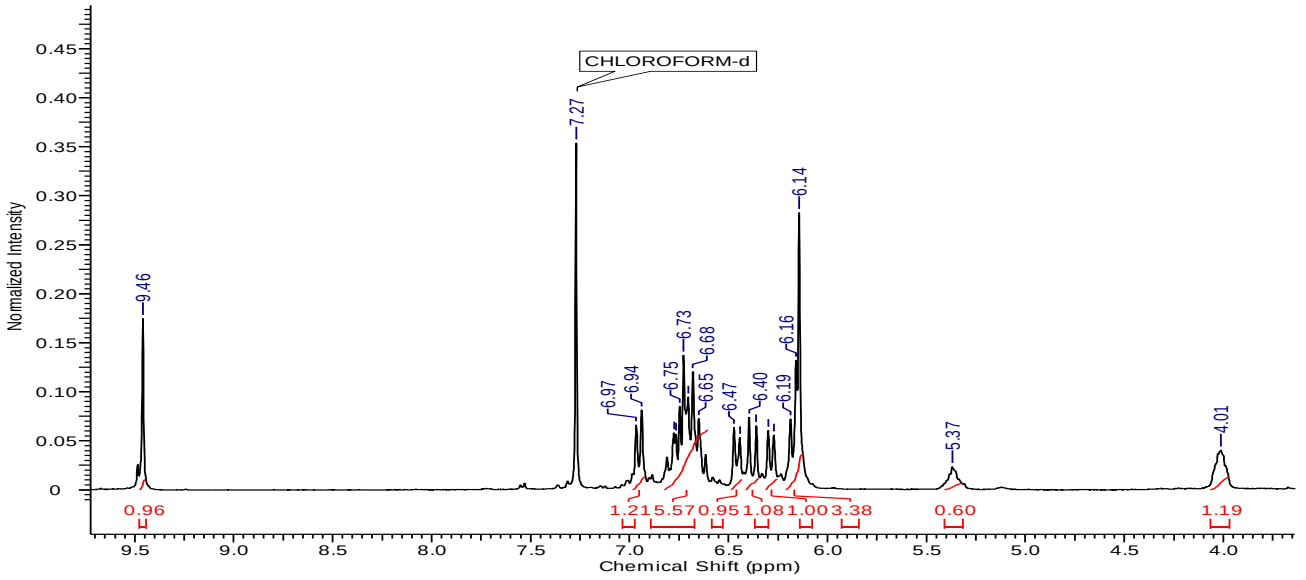

Fig. A5. ^{1}H NMR spectrum of ß-citraurin

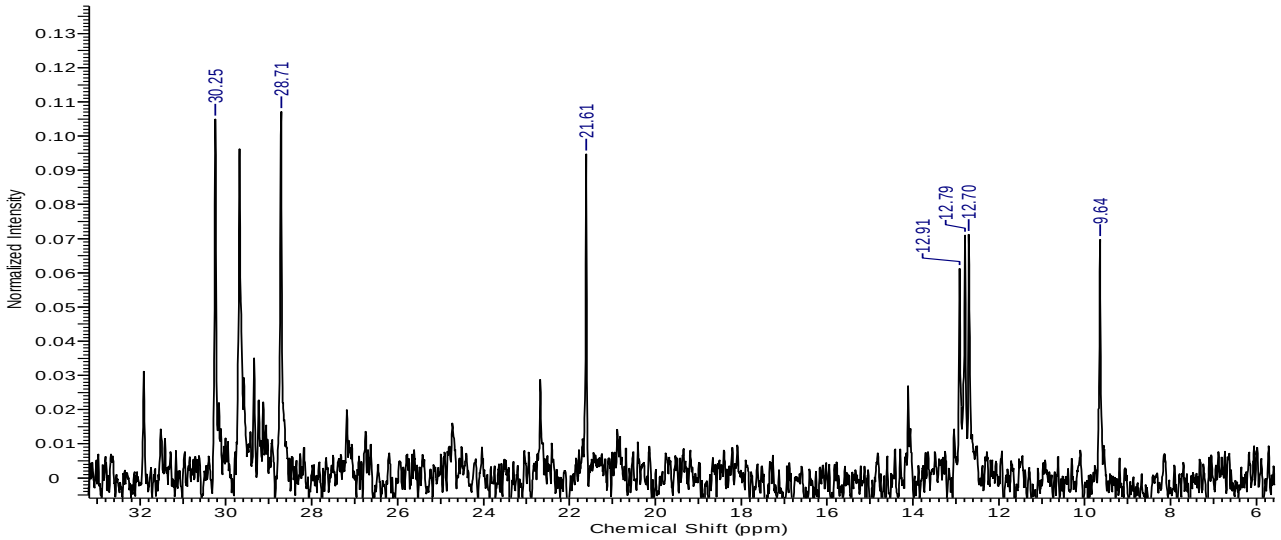

Fig. A6. ^{13}C NMR spectrum of ß-citraurin (6-32 ppm)

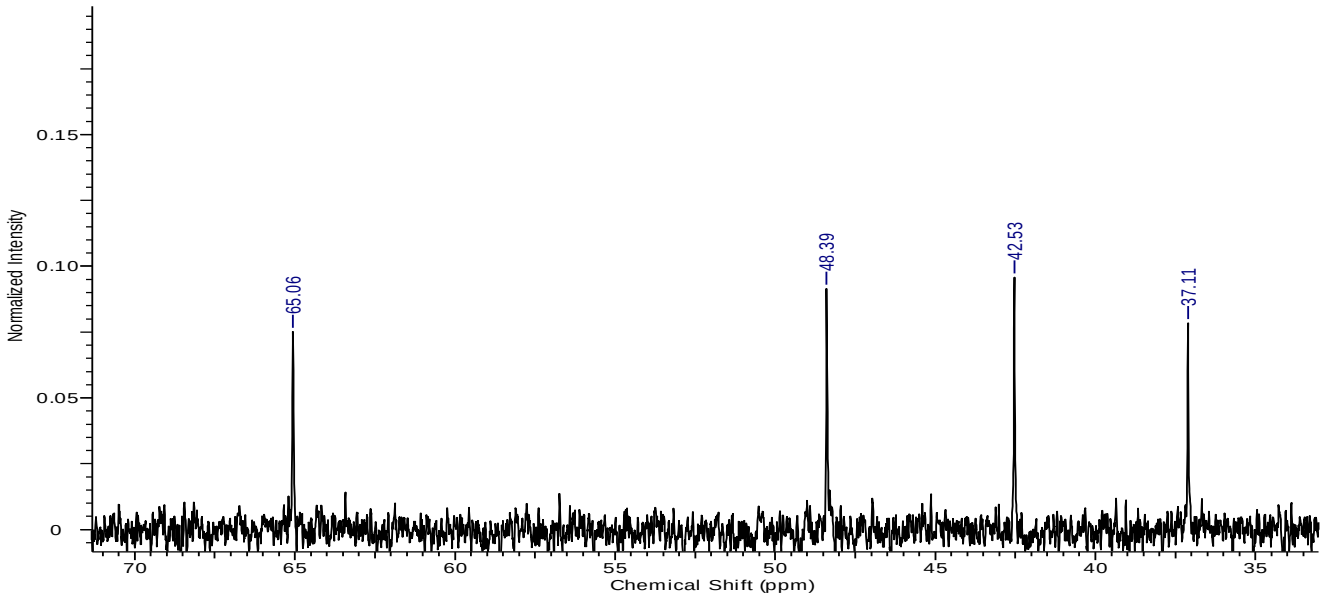

Fig. A7. ^{13}C NMR spectrum of ß-citraurin (33-70 ppm)

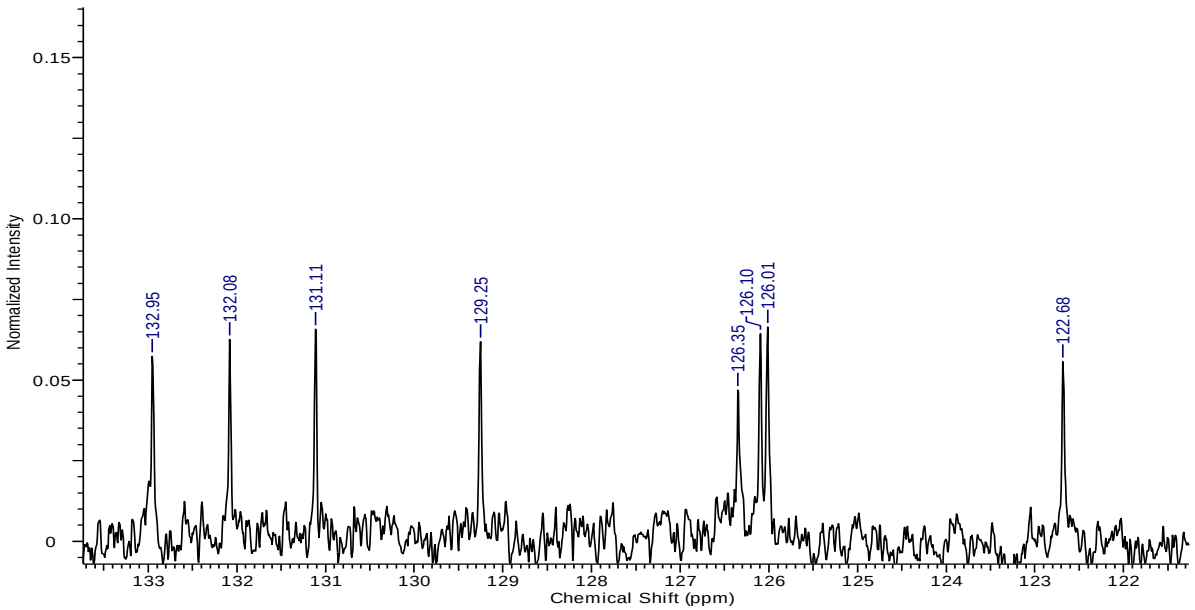

Fig. A8. ^{13}C NMR spectrum of ß-citraurin (120-135 ppm)

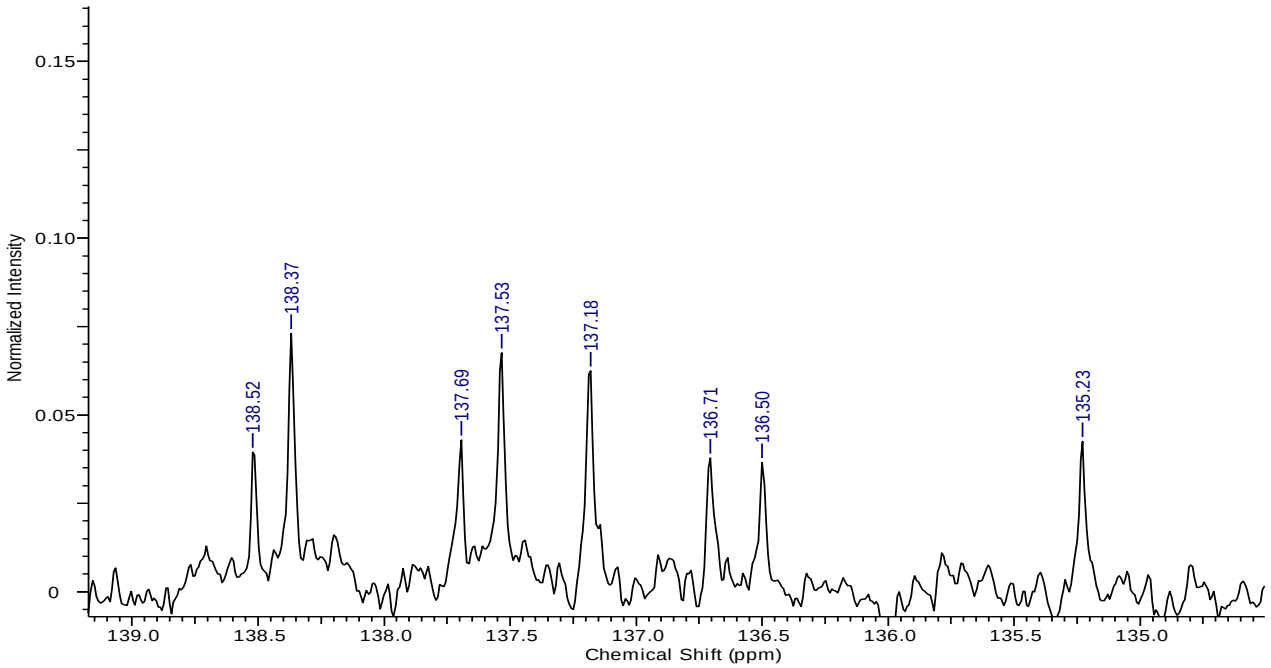

Fig. A9. ^{13}C NMR spectrum of β-citraurin (134-139 ppm)

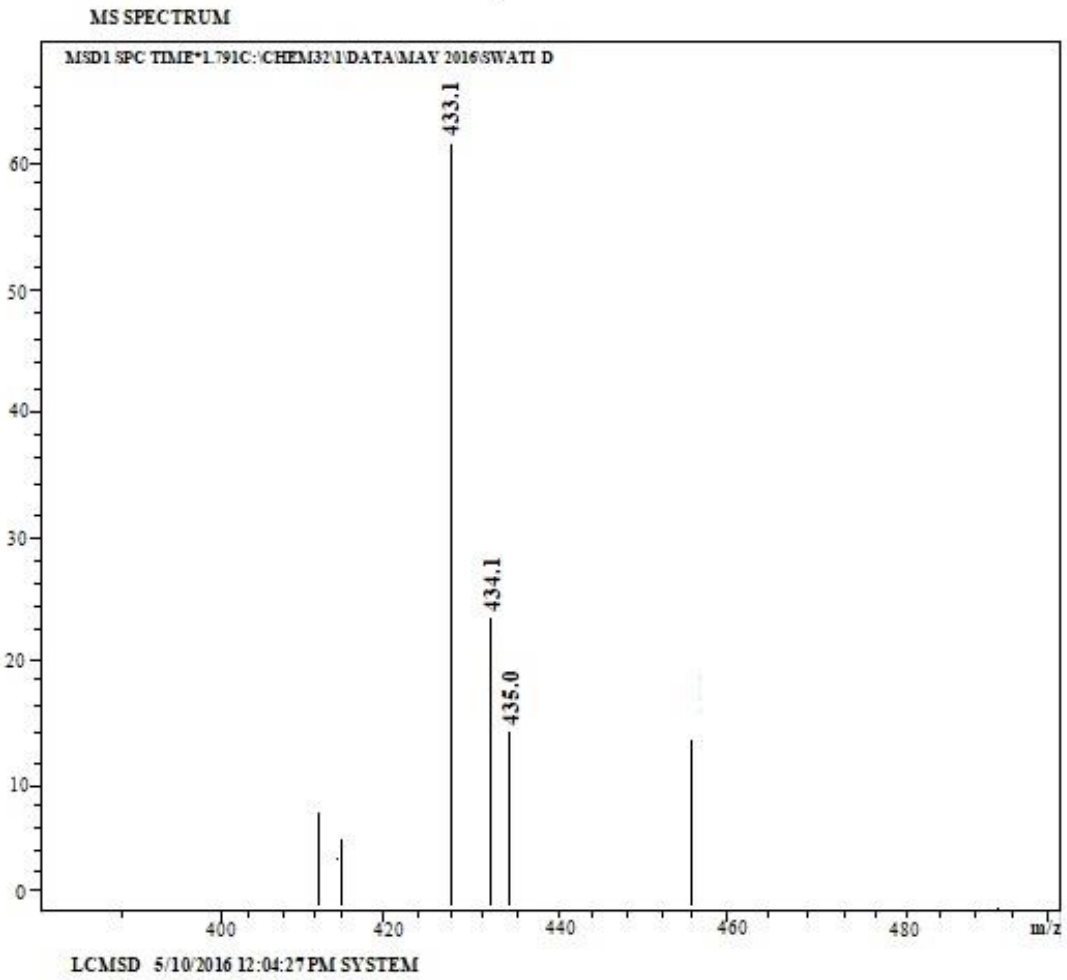

Fig. A10. Mass spectrum of β-citraurin

Tables

Table A1. ^{1}H NMR and ^{13}C NMR Chemical Shift Data (ppm) Obtained For ß-sitosterol Are Presented Alongside The Literature Values (Chaturvedula, V. S. P., & Prakash, I., 2012) For Comparison [19]

Position	^{1}H NMR δH (*J* in Hz)	Carbon Number	Literature Value of ^{13}C	Obtained Value of ^{13}C
1		1	37.5	38.77
2		2	31.9	31.95
3	3.53 (tdd 1H, *J*=4.5, 4.2, 3.8 Hz)	3	72.0	76.75
4		4	42.5	41.52
5	5.36 (t, 1H, *J*=6.4Hz)	5	140.9	140.27
6		6	121.9	121.72
7		7	32.1	32.65
8		8	32.1	32.78
9		9	50.3	50.44
10		10	36.7	35.38
11		11	21.3	22.71
12		12	39.9	39.71
13		13	42.6	42.08
14		14	56.3	55.17
15		15	26.3	26.66
16		16	28.5	28.74
17		17	56.3	55.13
18		18	36.3	36.66
19	0.94(d 3H, J=6.5Hz)	19	19.2	18.35
20		20	34.2	34.28
21		21	26.3	26.70
22		22	46.1	46.83
23		23	23.3	22.71
24	0.86 (t 3H, J=7.2Hz)	24	12.2	13.99
25		25	29.4	29.60
26	0.85 (d 3H, J=6.4Hz)	26	20.1	20.00
27	0.83 (d 3H, J=6.4Hz)	27	19.6	18.55
28	0.68 (s, 3H)	28	19.0	18.31
29	1.01 (s, 3H)	29	12.0	13.99

Table A2. ^{1}H NMR and ^{13}C NMR Peak Data For ß-citraurin

Position	^{1}H NMR δ_H (*J* in Hz)	^{13}C NMR δ_c
1	-	37.11
2	1.80, m	48.39
3	4.01, m	65.06
4	2.37, m	42.53
5	-	126.01
6	-	137.69
7	6.77-6.65, m	126.35
8		138.37
9	-	135.23
10	6.14-6.19, m	129.25
11	6.44-6.47, d (12)	126.10
12	6.27-6.30, d (12)	137.53

13	-	136.50
14	6.14-6.19, m	145.94
15	6.36-6.40, d (15)	131.11
16	1.08, s	30.25
17	1.08, s	28.71
18	1.75, s	21.61
19	2.01, s	12.91
20	2.01, s	12.79
8’	9.46, s	194.58
9’	-	138.52
10’	6.97-6.94, d (11)	149.34
11’	6.77-6.65, m	122.68
12’		137.18
13’	-	136.71
14’	6.14-6.19, m	132.95
15’	6.77-6.65, m	132.08
19’	1.91, s	9.64
20’	1.99, s	12.70
3-OH	5.37	-

Author Contributions: Conceptualization, S.P.; and V.P.C.; methodology, S.P.; validation, V.P.C., B.K.; and R.J.; investigation, S.P.; resources, V.P.C.; data curation, S.P.; and D.J.; writing—original draft preparation, S.P.; and D.J.; writing—review and editing, D.J.; and R.J.; visualization, S.P.; D.J.; and R.J.; supervision, V.P.C.; project administration, B.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

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