DOI: https://doi.org/10.31351/vol31issSuppl.pp54-61

Phytosterol Profile in Iraqi *Lactuca serriola*_after Purification and Isolation by Combiflash and HPLC (Conference Paper)#

Murtadha Saleh Hussien*,1 and Ayad A. Al-Hamashi**

 # 10th scientific conference sponsored by College of Pharmacy, University of Baghdad 2-3 June 2022
*Pharmacognosy and Medicinal Plants, College of Pharmacy, University of Baghdad, Baghdad, Iraq
**Pharmaceutical Chemistry, College of Pharmacy, University of Baghdad, Baghdad, Iraq

Abstract

One of these plants utilized in traditional medicine is *Lactuca seriolla* Linn., which belongs to the Asteraceae family. It goes by a variety of common names in the world, including prickly lettuce, wild lettuce, jagged lettuce, and Kahu and khas. The work aimed to isolate and characterize some bioactive constituent(s) from the aerial part of *Lactuca serriola* utilizing Combiflash NEXTGEN and high-performance liquid chromatography (HPLC). *Lactuca serriola* (aerial part) was extracted with 80% ethanol, then fractionated with hexane. Then 250 mg of hexane extract was mixed with 4 g of silica gel and loaded in cartilage, then bounded to the gold column of combi flash using a solvent system comprised of ethyl acetate: n-hexane (10% ethyl acetate 90% hexane v/v) to elute the column. The fractions resulting were analyzed by thin layer chromatography and high-performance liquid chromatography (HPLC). white needle-like crystalline substances were isolated via combi flash column chromatography. The isolated product was analyzed by thin layer chromatography and high-performance liquid chromatography (HPLC). β-sitosterol (11 mg) and stigmasterol (7 mg) were obtained from 250 mg of hexane fraction.

Keywords: Combiflash column chromatography, HPLC, *Lactuca serriola*, β-sitosterol, Stigmasterol.

الكشف عن الستيرويدات النباتية في نبات الخس البري العراقي بعد التنقية والعزل بواسطة(الفوتوغرافيا السائلة عالية الأداء , الفلاش كروماتوغرافي) (بحث مؤتمر)#

مرتضى صالح حسين *،١ و اياد عبد علي الهماشي**

 # المؤتمر العلمي العاشر لكلية الصيدلة، جامعة بغداد ٢ – ٣ حزيران٢٠٢٢
*فرع العقاقير والنباتات الطبية، كلية الصيدلة، جامعة بغداد، بغداد، العراق
** فرع الكيمياء الصيدلانية، كلية الصيدلة، جامعة بغداد، بغداد، العراق

الخلاصة

أحد هذه النباتات المستخدمة في الطب التقليدي هو *Lactuca seriolla Linn*. ، الذي ينتمي إلى العائلة النجمية . يُطلق عليه مجموعة متنوعة من الأسماء الشائعة في العالم، بما في ذلك الخس الشائك، والخس البري ، والخس المسنن ، و Kahu و khas. يهدف العمل إلى عزل وتوصيف بعض المكونات النشطة بيولوجيًا من الجزء الهوائي من *Lactuca serriola* باستخدام Combiflash NEXTGEN والكروماتوغرافيا السائلة عالية الأداء (HPLC) . تم استخلاص *Lactuca serriola* (جزء هوائي) مع ٨٠٪ إيثانول بواسطة soxhlet، ثم تجزئته مع الهكسان. ثم تم خلط ٢٥٠ مجم من مستخلص الهكسان مع ٤ جم من هلام السيليكا وتم تحميلها في cartilage ، ثم تم ربطها ب gold column من فلاش كومبي . باستخدام نظام مذيب يتكون من (10% ethyl acetate 90% hexane v/v) لأزل العمود. تم تحليل الاجزاء الناتجة عن طريق كروماتوجرافيا الطبقة الرقيقة والكروماتوجرافيا السائلة عالية الأداء .(HPLC) تم عزل المواد البلورية البيضاء الشبيهة بالإبرة عن طريق كروماتوجرافيا Combiflash NEXTGEN. تم تحليل المنتج المعزول بواسطة كروماتوجرافيا الطبقة الرقيقة والكروماتوجرافيا السائلة عالية الأداء .(HPLC) تم الحصول على بيتا-سيتوستيرول (١١ مجم) وستيجماستيرول (٧ مجم) من ٢٥٠ مجم من جزء الهكسان.

الكلمات المفتاحية :الخس البري ، الفصل بواسطة Combiflash NEXTGE ، التحليل بواسطة (HPLC) ، بيتا-سيتوستيرول ، ستيجماستيرول.

Introduction

Plants have been utilized as a medicine source for thousands of years for most of the world's population [(1)]. Early civilizations in China, India, and the Near East used medicinal plants as a source of sickness relief [(2,3)]. Per the World Health Organization (WHO), traditional medicines are used by more than 80% of the world's population for primary health care. The medical value of plants has related to the presence of chemical compounds that have various pharmacological effects on the human body. Alkaloids, flavonoids, tannins, and phenolic compounds are examples of critical bioactive molecules [(4)]. Botany, chemistry, and pharmacology are all involved in researching medications derived from plants. Botany deals with the classification and cultivation of plants. Isolation, identification, and quantification of components in plant materials are all part of chemical characterization. Pharmacology studies plant components' physiological effects on animal and human cells.

[1]Corresponding author E-mail: mortadhasalih568@gmail.com
Received:28 /6 /2022
Accepted:5 /9 /2022

Taxol/paclitaxel, vinblastine, vincristine, etoposide, atropine, morphine, ergotamine, physostigmine, digoxin, and other critical medications utilized in modern medicine have all emerged from medicinal plant research. Nowadays, linking folkloric uses of plants and expert knowledge of herbal medicine to modern research operations provides a noveltechnique that significantly increases the rate of drug discovery over random collection [(5)]. Asteraceae species involve many phytochemical components such as phytosterol, essential oils, lignans, saponins, polyphenolic compounds , phenolic acids, sterols, and polysaccharides that have medicinal effects [(6)]. *Lactuca seriolla* is one of these wild plants belonging to the Asteraceae family [(7)]. *Lactuca seriolla* L in, a member of the Asteraceae family, is an example of a traditional medicinal plant . Prickly lettuce, wild lettuce , Jagged lettuce , Kahu, and Kha are some of the common names used in Iraq . *Lactuca serriola* is a member of the Asteraceae family that grows in arid conditions in the northern hemisphere and has a milky sap that oozes freely from wounds. When this comes into touch with air, it hardens and dries the sap contains Lactucarium, which has antispasmodic, digestive, diuretic, hypnotic, tranquilizer , and sedative properties [(8)].

Phytosterols (also known as plant sterols) are essential components of plant cells. Squalene, a part of the triterpene family, is the source of these chemicals. This set of molecules has 28 or 29 carbon atoms and one or two carbon-carbon double bonds, contributing to the wide range of natural structures. More than 250 compounds have been discovered so far. *β*-sitosterol (C-29), campesterol (C-28), and stigmasterol (C-29) are the most commonly encountered phytosterols, accounting for up to 98 percent of phytosterol dietary intake in humans (Figure1) [(9-11)].'

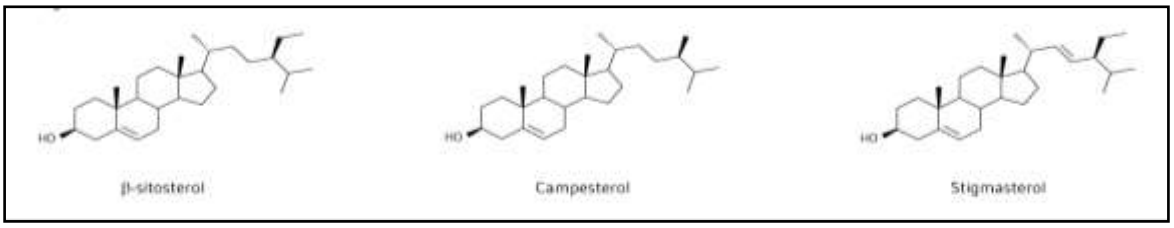

Figure 1. Chemical structure of selected phytosterols.

These substances are consumed by people and may have a substantial biological impact on maintaining and improving health [(12)]. Phytosterols compete with and block cholesterol absorption in the intestine because of their structural similarity to cholesterol, lowering LDL-C blood levels. Agren et al. discovered that individuals with rheumatoid arthritis who consumed 732 mg of phytosterols daily had decreased LDL-C and total cholesterol levels [(13)]. In addition to the physiological functions listed above, phytosterols perform a variety of essential roles in human health promotion. They can, for example, improve insulin resistance [(14)] and lipid metabolism [(15)], as well as reduce the risk of cancer [(16)], Alzheimer's disease, and atherosclerosis-related CVDs [(17)]. In this work, we isolated and characterized several bioactive constituent(s) from the aerial part of *Lactuca serriola* using flash chromatography and HPLC.

Materials and Methods

Collection of plant materials.

The *Lactuca serriola* plant materials (aerial part) were gathered at AL-Diwaniyah city, 180 km south of Baghdad, between September and November 2021. The plant was identified, validated, and authenticated by Prof. Dr. Sukaena Abbas/ Department of Biology/ College of the Science /University of Baghdad. The plant was cleaned, allowed to dry in the shade at room temperature, ground up using an electrical miller, and weighed [(18)].

Extraction method (a hot method by soxhlet)

A thimble was used for packaging 100 g of dried plant material (Aerial portion). A round flask containing one liter of 85 percent ethanol was then filled, and the thimble was placed in a Soxhlet extractor. Approximately 22 hours at 70 C° were spent extracting the sample completely to exhaustion. Using a rotary evaporator, the alcoholic extract was concentrated under reduced pressure, condensed to about 13 g, and then combined with 250 ml of distilled water to create a crude fraction [(19)].

Fractionation of extract

A crude fraction of 13 g was partitioned with solvents (differ in polarity) like Hexane, chloroform, ethyl acetate, and n-butanol (3x100 ml) for each fraction. The first three fractions dried over anhydrous sodium sulfate with except n-butanol fraction. The dried fractions were weighted and assigned for further analysis [(20)].

Chemical identification of phytosterol

Two types of chemical tests were used to recognize phytosterol compounds.

1-Liebermann-Burchard test: A small amount of plant hexane extract was dissolved in 5 ml of chloroform, and the chloroform layer was then dried over anhydrous sodium sulfate. The mixture was then combined with ten drops of acetic anhydride

and two drops of concentrated sulphuric acid. As oxidation proceeds in stages, the color is bluish green, indicating the existence of the steroidal nucleus.

2-Salkowski reaction: 2 ml of chloroform was used to dissolve a little amount of the hexane plant extract (F1), and then 3 ml of concentrated H_2SO_4 was added slowly. Due to the oxidation reaction, a reddish-brown hue was produced [21].

Preliminary identification of phytosterol (stigmasterol and β-sitosterol) by using TLC

A small amount of the hexane extract was diluted with chloroform, and the resulting solution was spotted onto TLC plates that had been coated. The TLC plates were developed using particular solvent systems, examined individually with U.V. light at 236 nm, and then sprayed with the Vanillin-H_2SO_4 reagent [22].

Isolation of proposed detected phytosterol by combi flash NEXTGEN column chromatography from hexane fraction

The purification process of flash chromatography is straightforward and requires little method development. With the Teledyne ISCO CombiFlash NextGen flash chromatography system, you can automate processes with high productivity, create gradients with programmable parameters, separate peaks using U.V. light, and automatically identify columns and collecting tube racks (system dependent). Its small size makes it an excellent personal system and makes it suitable for use in chemical hoods and other cramped indoor areas. In some settings, use on an open bench is permitted with the addition of an optional fraction collection enclosure. In comparison to conventional gravity-based column chromatography, separations that use a gas pressured solvent reservoir to speed up solvent flow can be completed more quickly**.** Depending on spots that appear in TLC after the use proper solvent system (10% ethyl acetate and 90% n-hexane v/v), 250 mg of hexane extract mixed with 4 g silica gel, and This sample on silica is then poured into an empty cartridge, topped with a frit and loaded onto the system, combi flash using a solvent system comprised of ethyl acetate: n-hexane (10% ethyl acetate and 90% n-hexane v/v) to elute the column to obtain 28 fractions of 30 ml each. Collected fractions were spotted on a TLC plate (Figure 3)[23].

HPLC analysis

The hexane fraction components were separated utilizing the HPLC system of Knauer Germany. The active components were compared to standard materials through U.V./V is absorption analysis. The retention time for the sample and Standard were identified. The integrated peak area was used to assess the sample quantity, and the calibration curve content was derived by plotting the sample peak area against the concentration of the appropriate standard sample. The C18 HPLC column (250 × 4.6) 5 m particles from the USA's Water Corporation were used to achieve the separation. The mobile phase used for phytosterol detection was MeOH and acetonitrile of 30:70 MeOH and acetonitrile, and the solvent system gradient was gradually increased to 0:100. The elution was sustained for 10 minutes, followed by 30:70 for 15 minutes in a flow rate of 1 ml/min. The wavelength of 210 nm was used for U.V. detection [24].

	Component	Model	Company and origin
1	Binary high-pressure gradient pump	P6.1L	Knuaer, Germany
2	Diode array detector	DAD 2.1L	Knuaer, Germany
3	Sample loop (20µl) and injector	D1357	Knuaer, Germany
4	Analyses and system control software	Claritychrom, V 7.4.2.107	Dataapex, Czech Republic

Result and Discussion

Chemical tests were employed to identify phytosterol compounds in *Lactuca serriola*, and the findings are shown in Table 1. The Libermann-Burchard reaction and Salkowski reaction tests confirmed that steroids were present in the aerial part of *Lactuca serriola* [25].

Table 1. Result of the chemical tests for hexane fraction

Plant Parts	Libermann-Burchard reaction	Salkowski reaction
The aerial part of a plant	+	+

Stigmasterol and β-sitosterol in the aerial portion of *Lactuca serriola* hexane extract were detected using TLC. The spot color and the R_f value for stigmasterol and β-sitosterol were identical because the two compounds' structures are similar and only differ in the presence of a C22=C23 double bond in stigmasterol and a C22-C23 single bond in β-sitosterol (Figure 2) [26].

Figure 2. Thin layer chromatogram for Hexane fraction(h) with beta-sitosterol(b) and stigma sterol(s) std. Using silica gel GF_{254nm} as a stationary phase developed in Hexane: ethyl acetate 9:1 as mobile phase. They were visualized by 5% H2SO4 spray reagent followed by heating for 5-10 minutes at 110 C°.

Table 2. R_f value of phytosterol obtained from hexane fraction of Iraqi *Lactuca serriola* compared to stigmasterol and b-sitosterol standard.

Standard	Solvent systems	R_f (retardation factor) value of Standard	R_f (retardation factor) value of compound L2
Stigmasterol	Hexane: ethyl acetate 9:1	0.26	0.25
b-sitosterol	Hexane: ethyl acetate 9:1	0.25	0.25

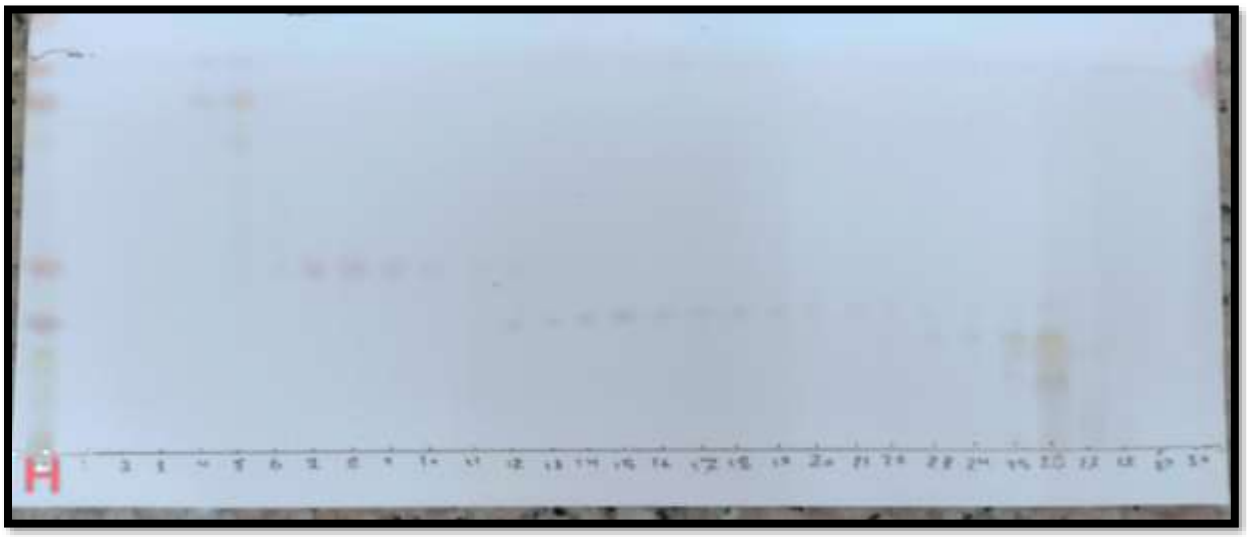

Figure 3. **Thin layer chromatogram for Hexane fraction (H)with fractions obtained by combi flash column chromatography after purification hexane fraction. Using silica gel GF_{254nm} as a stationary phase developed in (Hexane: acetone 8:2) as mobile phase. Visualized by 5% H2SO4 spray reagent followed by heating for 5-10 minutes at 110 C°.**

The combi flash fractions were eluted through HPLC for Further separation. The compounds were compared with the respected standard material. HPLC data indicated the presence of phytosterol. The content of the sample was computed using the calibration curve by plotting the peak area versus the concentration of the appropriate reference sample. The sample's quantity was determined by measuring the integrated peak area. The HPLC analysis indicated that the combi flash fractions (12-20) contained phytosterol. On the other hand, combi flash fractions 19 and 20 contain stigmasterol only. And fractions 12,16 have a mixture of sitosterol and stigmasterol (Figure 4).

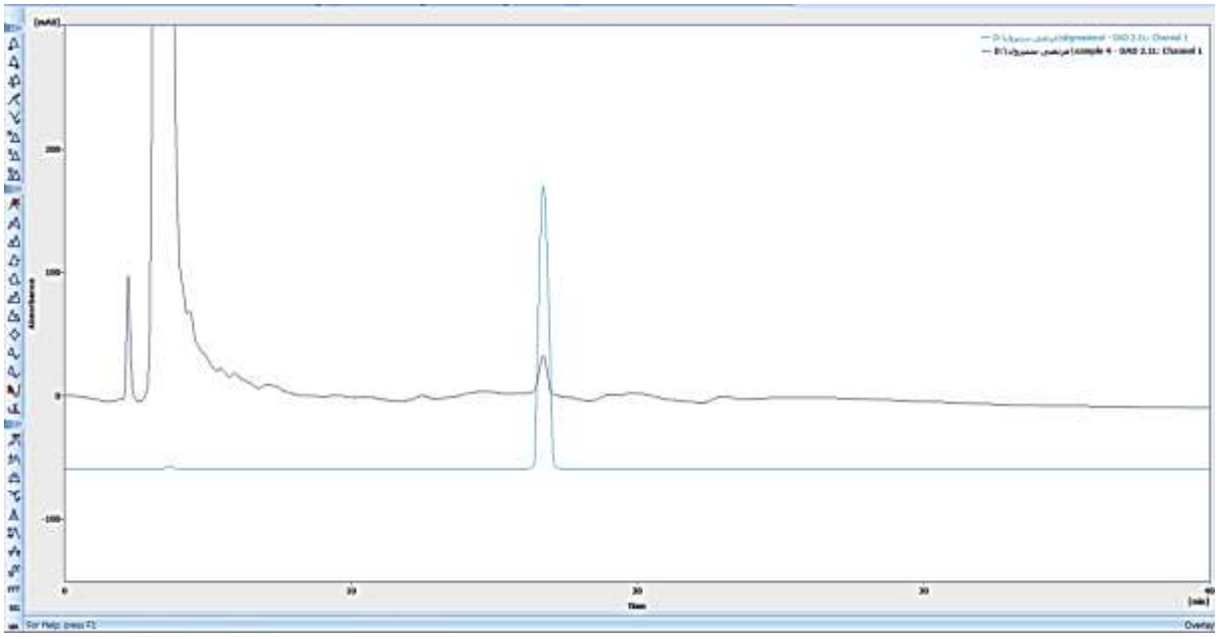

Figure 4. HPLC chromatogram analysis fractions contain stigmasterol [Standard stigmasterol (-) sample (-)]. The retention time for isolated compound and stigmasterol standard is identical (16.9).

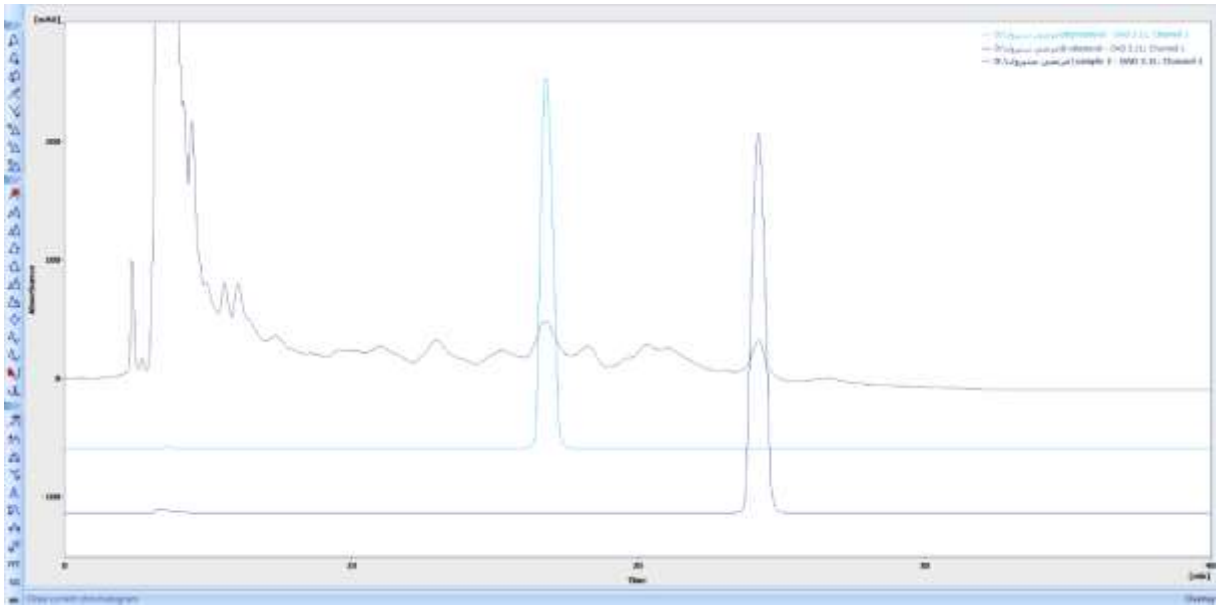

Figure 5. HPLC chromatogram analysis fractions contain β-sitosterol and stigmasterol(sample, Standard). The retention time for isolated compound & β-sitosterol standard is identical (24.1).

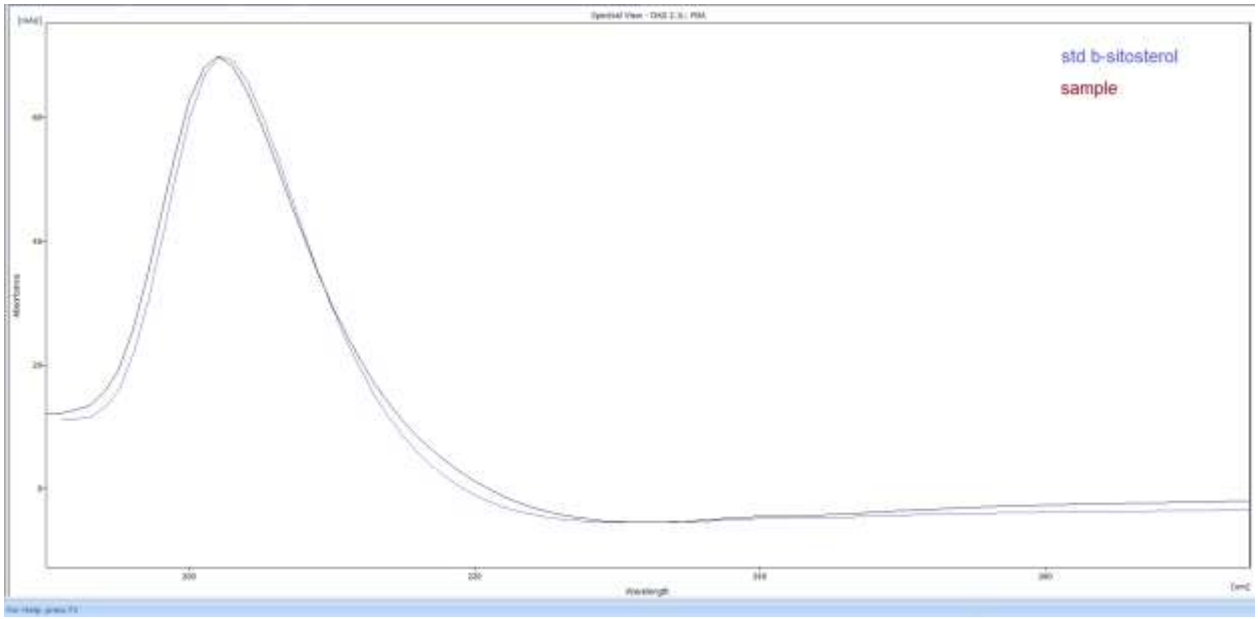

Figure 6. U.V. spectrum view using the diode array detector (DAD) for β-sitosterol peak. STD, B-sitosterol standard absorbance(-). Sample(-),β-sitosterol peak absorbance at λ 210 nm.

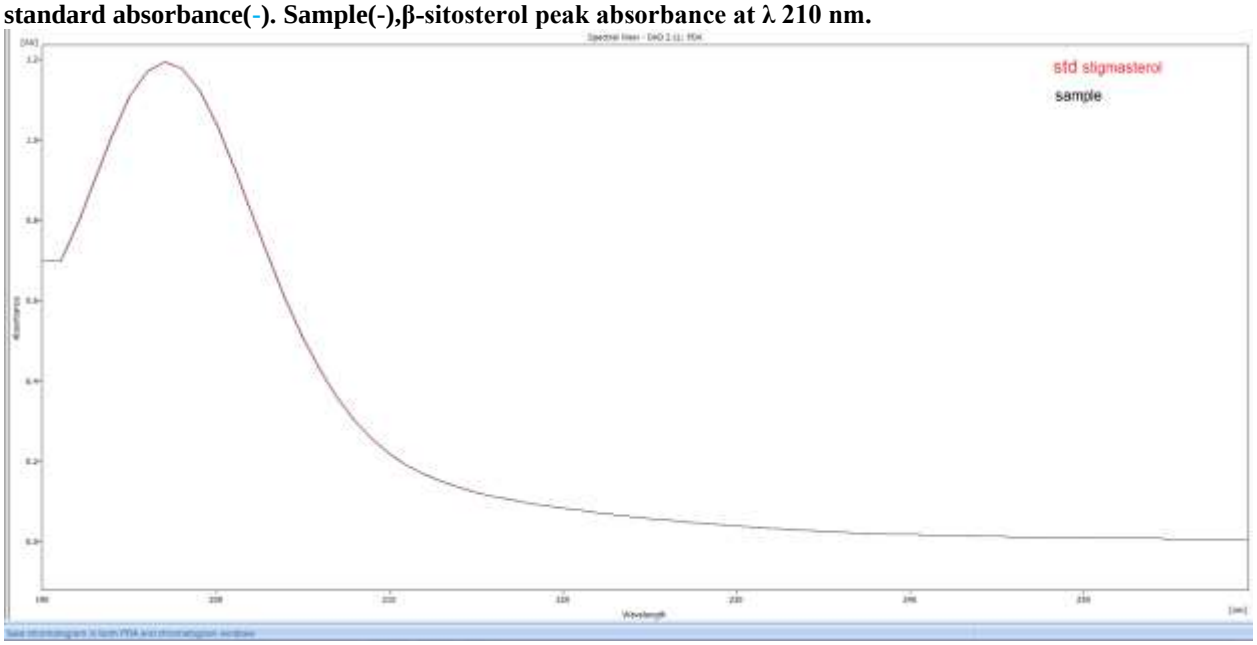

Figure 7. U.V. spectrum view using the diode array detector (DAD) for stigmasterol peak. STD, stigmasterol standard absorbance(-). Sample(-), stigmasterol peak absorbance at λ 210 nm.

For quantitative analysis, the calibration curve was plotted using the area under the curve (AUC) versus four concentration levels of stigmasterol and β-sitosterol standards from which the concentration of the proposed stigmasterol and β-sitosterol (L1 and L2) in the hexane fraction.

The concentration was calculated applying a straight line equation, as illustrated in Figures 6 and 7. The calculated concentration of proposed stigmasterol and β-sitosterol (L1 and L2) in the Iraqi *Lactuca serriola* is shown in Table 2.

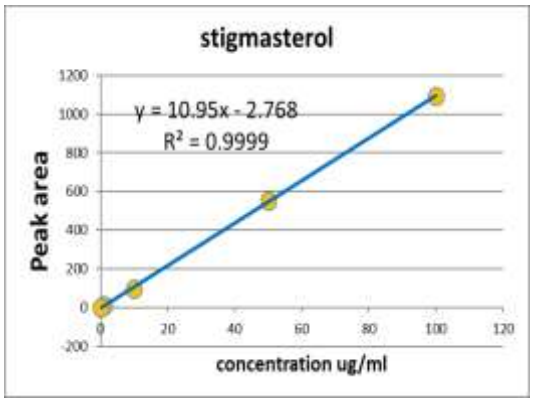

Figure 7. The calibration curve of Stigmasterol

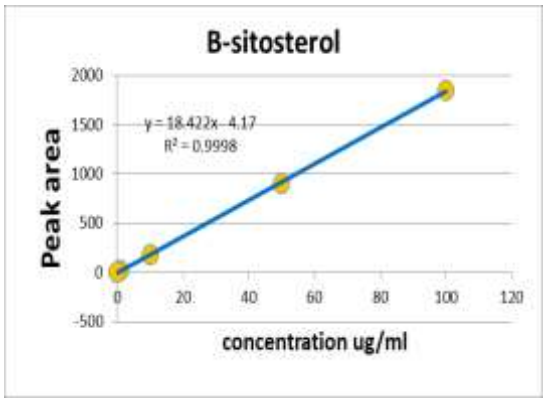

Figure 8. calibration curve of β-sitosterol

Table 2. The quantity of the plant's hexane fraction that contains the predicted stigmasterol and β-sitosterol (L1 and L2).

Samples	peak area	µg/ml of fraction	µg/mg plant material
Stigmasterol	163.299	4.55566373	91.11327459
β-sitosterol	268.028	4.064778919	81.29557838

Conclusion

A rapid and effective method for isolating and purifying major plant sterol from *Lactuca serriola* was performed. This method could provide reference substances for further phytochemical studies. To the best of our knowledge, this is the first report on separating phytosterol in medical plants using the flash column method in Iraq.

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