Extraction and Isolation of β-sitosterol from Iraqi Wild *Lycium barbarum* by Different Techniques (Probe and Bath Ultrasound, HPTLC and PHPLC)

Thukaa Z. Abdul-Jalil[*,1], Ahmed A.Hussein[**] and Kawkab Y. Saour[***]

[*]Department of Pharmacognosy, College of Pharmacy, University of Baghdad, Baghdad, Iraq.
[**]Department of Pharmaceutics, College of Pharmacy, University of Baghdad, Baghdad, Iraq.
[***] Department of Pharmaceutical Chemistry, College of Pharmacy, University of Baghdad, Baghdad, Iraq.

Abstract

Lycium barbarum (Awsaj) is a plant belong to family Solanaceae serves as a good source of bioactive compounds like phytosterols which have many important biological activity. Literature survey available so far revealed that there was no studies about Iraqi wild Awsaj phytosterols especially ß-sitosterol, there for the objective of this study was to examine the efficiency of ultrasound assisted extraction (probe and bath) as compared to the conventional (Soxhlet) extraction method for extraction of phytosterols especially β-sitosterol from fruits, leaves, stems and roots of Iraqi wild Awsaj plant. This goal was achieved by comparing the extraction mass yield, also by a quick and easy approach for identification and quantification of bioactive β-sitosterol in four parts of Awsaj using high performance thin layer chromatography (HPTLC) which confirm the presence of β-sitosterol in four parts of Iraqi Awsaj plant and the results clarify that the root part of Iraqi Awsaj plant extracted by probe UAE give the highest concentration (0.610 mg/ml) of B-sitosterol followed by fruits (0.465mg/ml), leaves(0.405mg/ml) and the least was stems(0.275mg/ml); according to these; isolation and purification of ß-sitosterol from petroleum ether fraction of Awsaj roots was done by preparative HPLC. The isolated compound (β-sitosterol) was identified by measuring melting point, FT-IR, TLC and preparative HPLC.

Keywords: Awsaj, Ultrasound assisted extraction (UAE), β-sitosterol, high performance thin layer chromatography (HPTLC), Preparative high performance liquid chromatography (PHPLC).

استخلاص وفصل مركب بيتاستوستيرول من نبات العوسج البري العراقي بواسطة تقنيات مختلفة (مسبارد حوض الامواج الفوق الصوتية ، الفصل اللوني للطبقة الرقيقة ذات الانجاز العالي والفصل اللوني السائل ذو الانجاز العالي)

ذكاء زهير عبد الجليل [*,1] ، احمد عباس حسين[**] و كوكب يعقوب ساعور [***]

[*]فرع العقاقير والنباتات الطبية ، كلية الصيدلة ، جامعة بغداد ، بغداد ، العراق .
[**] فرع الصيدلانيات ، كلية الصيدلة ، جامعة بغداد ، بغداد ، العراق .
[***] فرع الكيمياء الصيدلانية ، كلية الصيدلة ، جامعة بغداد ، بغداد ، العراق .

الخلاصه

العوسج (*Lycium barbarum*) هو نبات من العائله الباذنجانيه ويعتبر مصدر جيد للكثير من المركبات مثل الستيرويدات النباتيه ذات الفعاليه الاحيائيه. و نظرا لعدم وجود دراسات حتى الوقت الحالي حول الستيرويدات النباتيه و خصوصا مركب بيتا-سيتوستيرول في النبات العراقي البري العوسج, لذلك اصبح هدف العمل في هذه الدراسه هو لفحص كفائة الاستخلاص بمساعدة الامواج فوق الصوتيه (بوساطة المسبار و الحوض) و مقارنته بطريقة الاستخلاص التقليدي بوساطة جهاز السوكسليت (Soxhlet)لاجل استخلاص الستيرويدات النباتيه و خصوصا البيتاسيتوستيرول من الثمار, الاوراق, السيقان و الجذور لنبات العوسج العراقي البري. هذا الهدف تم انجازه بمقارنة حصيلة الاستخلاص الكتلي و كذلك عمل مقارنة سريعه و بسيطه لغرض الكشف و التحديد الكمي لمركب البيتاسيتوستيرول في الاجزاء الاربعه لنبات العوسج باستعمال الفصل اللوني (كروماتوغرافيا) للطبقه الرقيقه ذات الانجاز العالي الذي اكد وجود البيتا-سيتوستيرول في الاجزاء الاربعه لنبات العوسج. اوضحت النتائج ان جذور النبات المستخلصه بوساطة مسبار الامواج فوق الصوتيه تحتوي على اعلى نسبة من البيتاسيتوستيرول (٠,٦١٠مغ\مل) يتبعها الثمار بنسبة (٠,٤٦٥مغ\مل) و الاوراق بنسبة (٠,٤٠٥مغ\مل) و اخرا السيقان بنسبة (٠,٢٧٥مغ\مل). وفقا لتلك النتائج تم فصل و تنقية مركب البيتا-سيتوستيرول من المستخلص اثير البترول لجذور العوسج باستعمال تقانة الفصل اللوني السائل ذات الانجاز العالي. كذلك استخدمت مجموعه من التقانات للتحقق من نوعية المركب المفصول (بيتا-سيتوستيرول) و درجة نقاوته و التي شملت قياس درجة الانصهار ,الاشعه تحت الحمراء, الفصل اللوني للطبقة الرقيقه و الفصل اللوني السائل ذات الانجاز العالي.

الكلمات المفاتيحيه: العوسج, الاستخلاص بمساعدة الامواج فوق الصوتيه ،البتا-سيتوستيرول ، الفصل اللوني للطبقه الرقيقه ذات الانجاز العالي، الفصل اللوني السائل ذات الانجاز العالي.

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[1]Corresponding author E-mail: aljas_dadid@yahoo.com
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Introduction

Nature has blessed mankind with a treasure of bioactive ingredients which are an emerging field in the context of health and nutrition. The American dietetic association (ADA) define the bioactive ingredients of food as a physiologically active constituent in food or dietary supplement derived from both animal and plant sources including those needed to meet basic human nutritional needs that have been demonstrated to have a role in health and safe of human consumption [1, 2] .

Phytosterols especially β-sitosterol is a predominant sterol in human herbal nutrition forming 65% diet contents can be considered as constituent of bioactive foods ,(figure 1) [3] .

Awsaj (*Lycium barbarum*), also known as Goji, is a shrub belong to Solanaceae family and is native to Asia, (figure 2) [4]. Awsaj has been used in Asian countries as a traditional herbal medicine and functional food which possess a large variety of beneficial effects, including antiaging activity [5], immune modulation [6], antioxidant[7], neuroprotective effect[8], anticancer[9], male fertility facilitatory action [10], lower blood pressure and blood cholesterol level[11] as well as hypoglycemic properties [12].

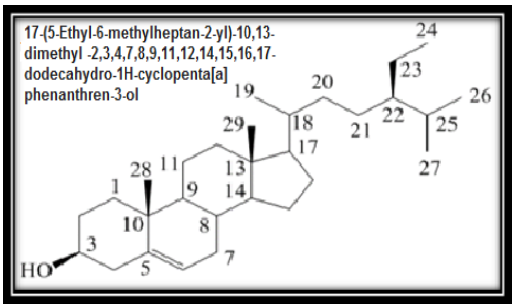


Figure (1): structure of β-sitosterol

Figure (2): Iraqi *Lycium barbarum* (Awsaj) plant

Literature survey available so far revealed that there are no study concerned with Awsaj ß-sitosterol in Iraq, therefore the objective of the present study is to extract ß-sitosterol from four parts of Iraqi wild Awsaj plant (fruits, leaves, stems and roots) then identification and quantification by high performance thin layer chromatography (HPTLC) further more isolation of ß-sitosterol from Awsaj by preparative high performance liquid chromatography (PHPLC).

Materials and Methods

Plant material

Four parts (fruits, leaves, stems and roots) of Awsaj (*Lycium barbarum L.),* which grows as a wild plant in Iraq, were collected in October and November 2016 from the University of Baghdad in Al-Jadiryia District, Baghdad, then authenticated by the herbarium of the Department of Biology, College of Science/University of Baghdad registered at BUH No. 50777. These parts were left to dry in shade then grinded using an electric blender, weighted and subsequently subjected to extraction procedure.

Extraction methods

Two different extraction methods were used; the first representing a classical conventional (Soxhlet) method (A) and the second is a simple, fast and nonconventional extraction (ultrasound assisted extraction UAE) method (B).

Method A: Approximately 250 gm of grounded fruits, leaves, stems and roots were subjected to extraction using a Soxhlet apparatus in which each part of Awsaj were extracted separately with 85% methanol, then the crude methanol extract of each part was filtered, concentrated under reduced pressure then suspended in distal water and partitioned with petroleum ether (B.P, 60-80 C°) three times. Later on the fractions of petroleum ether were concentrated to dryness over anhydrous sodium sulfate, weighted and subjected for identification of ß-sitosterol by HPTLC [13, 14] .

Method B: the UAE was done using probe ultrasonicator and bath ultrasonicator with 85% methanol was used as a solvent. The UAE was carried out under the following experimental conditions in case of probe ultrasonicator the plant material is in direct contact with ultrasonic waves, temperature 25 C° ,time 20 minutes, solid to solvent ratio 1:8 gm/ml and sonication frequency 20KHz and power of 60 watt. While in bath ultrasonicator the ultrasonic waves have to pass through the glass material in order to come in contact with plant material, temperature 50 C° ,time 60 minutes, solid to solvent ratio 1:8 gm/ml and sonication frequency 60KHz and power of 60 watt. In the current work, both instruments were used in order to study the effect of direct and indirect contact of ultrasonic waves on extraction of ß-sitosterol from four parts of Iraqi Awsaj plant. The same schematic procedure was used in conventional method (Soxhlet) was applied, in which the crude methanolic extract filtered, concentrated, suspended in distal water, partitioned by petroleum ether. Later on the fractions of petroleum ether were concentrated to dryness over anhydrous sodium sulfate, weighted and subjected for identification of β-sitosterol by HPTLC [15-18] .

Identification and quantification of ß-sitosterol by HPTLC

The sample solutions were spotted in the form of bands of width 8.0 mm with a Camag microliter syringe on precoated silica gel glass plate 60F254 (20 cm × 10 cm with 250 µm thickness; E. Merck KJaA) using a Camag Linomat 5 (Switzerland). A constant application volume of 4 µl was employed and space between two bands was 3.3 mm. The slit dimension was kept at 4.0mm × 0.3 mm and 20 mm/s scanning speed was employed and each track was scanned thrice and baseline correction was used. The mobile phase consisted of toluene – ethyl acetate - chloroform, in the volume ratio of 5:1:4 (v/v) and 10 ml of mobile phase was used per chromatography. Linear ascending development was carried out in 20 cm x 10 cm automatic developing chamber (Camag, ADC 2) saturated with filter paper Whatmann no.1 in the mobile phase. The optimized chamber saturation time for mobile phase was 5 min at room temperature (25 C° ± 2) at relative humidity of 50% ± 5. The length of chromatogram run was 7.5 cm. Later on to the scanning, TLC plates were dried for 5 min with the help of an air dryer. Densitometry scanning was performed with Camag TLC scanner in the reflectance absorbance mode at 196 nm and operated by Win CATS software using tungsten lamp. Subsequent to the development, TLC plate was sprayed with 5% sulphuric acid reagent followed by drying in oven at 110 C° for few min, all these steps was done at Baghdad collage of pharmacy [2, 19] .

Quantitative estimation of B-sitosterol was done by using calibration curve in which series of diluted solutions (0.5, 0.25, 0.125, 0.0625 mg/ml) were prepared from ß-sitosterol standard stock solution (0.5mg/ml) [20] .

Isolation and purification of ß-sitosterol by preparative HPLC

ß-sitosterol was isolated from petroleum ether fraction of Awsaj roots by preparative high performance liquid chromatography (PHPLC) using Mediterranean Sea 18s µm (15x2.12, ambient) column and methanol (HPLC grade) as mobile phase. The isocratic elution was carried out for 10 min at flow rate 25ml/min, injection volume was 2 ml/min and detection was recorded with UV detector at 196 nm. All these steps was done at college of pharmacy/Baghdad University. The isolated compound was recrystallized using hot methanol and compared with standard ß-sitosterol by different identification method including: [21, 22]

1. Thin layer chromatography (TLC) using the best mobile phase toluene – ethyl acetate - chloroform 5:1:4 and detection by spraying with 5% H2SO4 + heating at 110 C°
2. Melting point.
3. Fourier transforms infrared (FT-IR) spectroscopy (FTIR) in KBr disk using FT/IR 4200 Type A.
4. Preparative high performance liquid chromatography (PHPLC) using the previously mentioned mobile phase.

Results and Discussion

Plant extract yield comparison

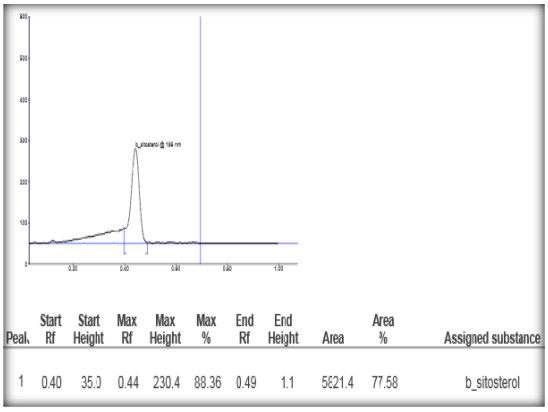
The results showed that probe UAE produced the higher yield in the extraction fraction followed by Soxhlet extraction fraction and finally bath UAE give the lower yield (table 1). The advantage of probe UAE is increased as it is considered the reduction of the extraction time and in the solvent consumption**.**

Table (1): Difference in extract content yield (gram of fraction / 250 gm) in different parts of Awsaj plant using different methods.

Parts of Awsaj	Soxhlet petroleum ether fraction	UAE petroleum ether fraction	
		Probe	Bath
Fruits	8.5	9.2	7.5
Leaves	10.5	11.3	9.4
Stems	3.2	3.8	2.9
Roots	5.3	6.9	4.8

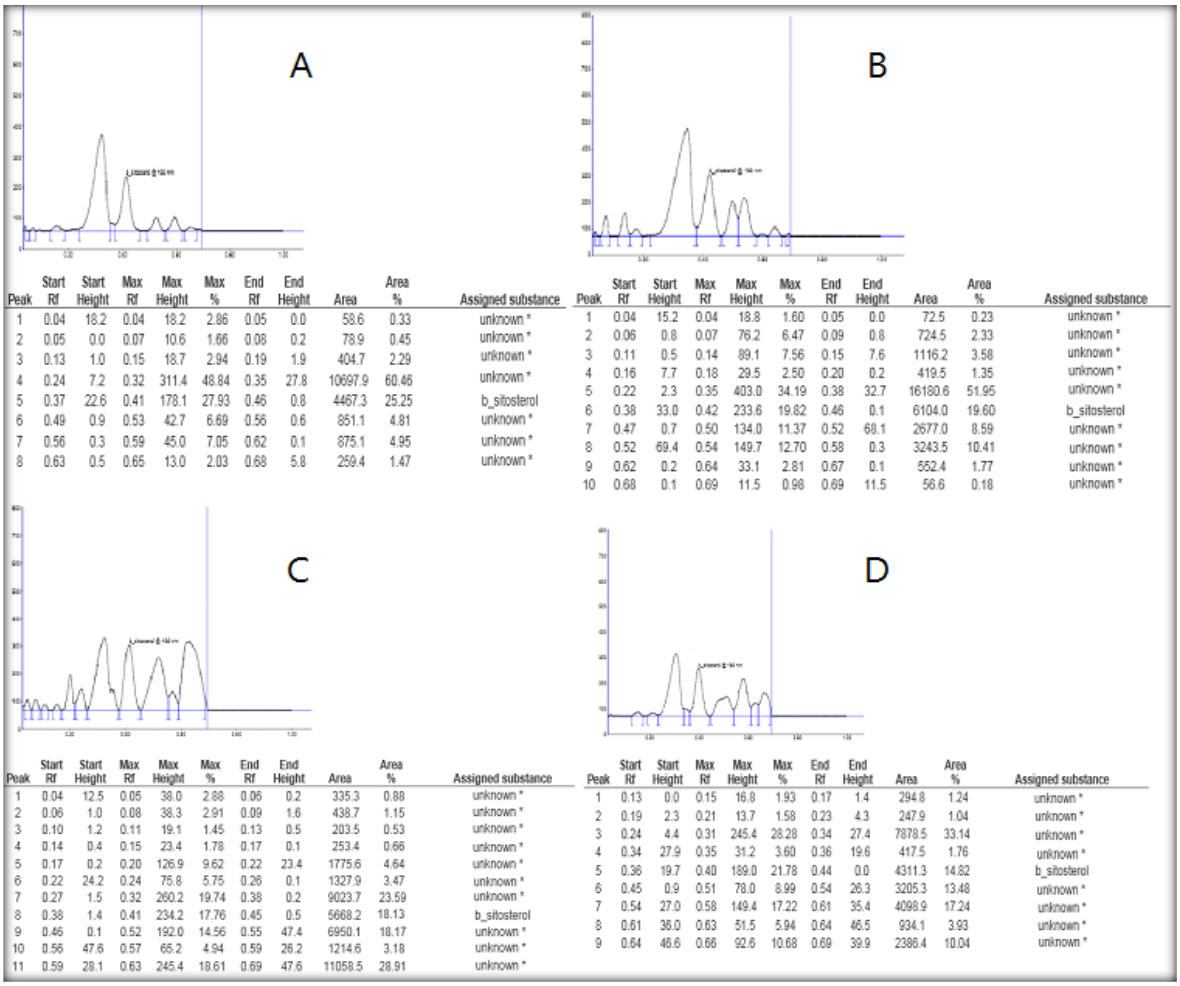
Identification and quantification of ß-sitosterol by HPTLC

The results indicated that the HPTLC method was developed for the first time for qualitative identification and quantitative estimation of β-sitosterol from four parts of Iraqi Awsaj plant in which qualitative identification was made by comparison of maximum retardation factor (R_f) and UV spectrum of petroleum ether fraction of each part with that of stander β-sitosterol as shown in figures 3-6.



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.40	35.0	0.44	230.4	88.36	0.49	1.1	5621.4	77.58	b_sitosterol

Figure (3): HPTLC chromatogram of ß-sitosterol standard



A

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.04	18.2	0.04	18.2	2.86	0.05	0.0	58.6	0.33	unknown *
2	0.05	0.0	0.07	10.6	1.66	0.08	0.2	78.9	0.45	unknown *
3	0.13	1.0	0.15	18.7	2.94	0.19	1.9	404.7	2.29	unknown *
4	0.24	7.2	0.32	311.4	48.84	0.35	27.8	10697.9	60.46	unknown *
5	0.37	22.6	0.41	178.1	27.93	0.46	0.8	4467.3	25.25	b_sitosterol
6	0.49	0.9	0.53	42.7	6.69	0.56	0.6	851.1	4.81	unknown *
7	0.56	0.3	0.59	45.0	7.05	0.62	0.1	875.1	4.95	unknown *
8	0.63	0.5	0.65	13.0	2.03	0.68	5.8	259.4	1.47	unknown *

B

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.04	15.2	0.04	18.8	1.60	0.05	0.0	72.5	0.23	unknown *
2	0.06	0.8	0.07	76.2	6.47	0.09	0.8	724.5	2.33	unknown *
3	0.11	0.5	0.14	89.1	7.56	0.15	7.6	1116.2	3.58	unknown *
4	0.16	7.7	0.18	29.5	2.50	0.20	0.2	419.5	1.35	unknown *
5	0.22	2.3	0.35	403.0	34.19	0.38	32.7	16180.6	51.95	unknown *
6	0.38	33.0	0.42	233.6	19.82	0.46	0.1	6104.0	19.60	b_sitosterol
7	0.47	0.7	0.50	134.0	11.37	0.52	68.1	2677.0	8.59	unknown *
8	0.52	69.4	0.54	149.7	12.70	0.58	0.3	3243.5	10.41	unknown *
9	0.62	0.2	0.64	33.1	2.81	0.67	0.1	552.4	1.77	unknown *
10	0.68	0.1	0.69	11.5	0.98	0.69	11.5	56.6	0.18	unknown *

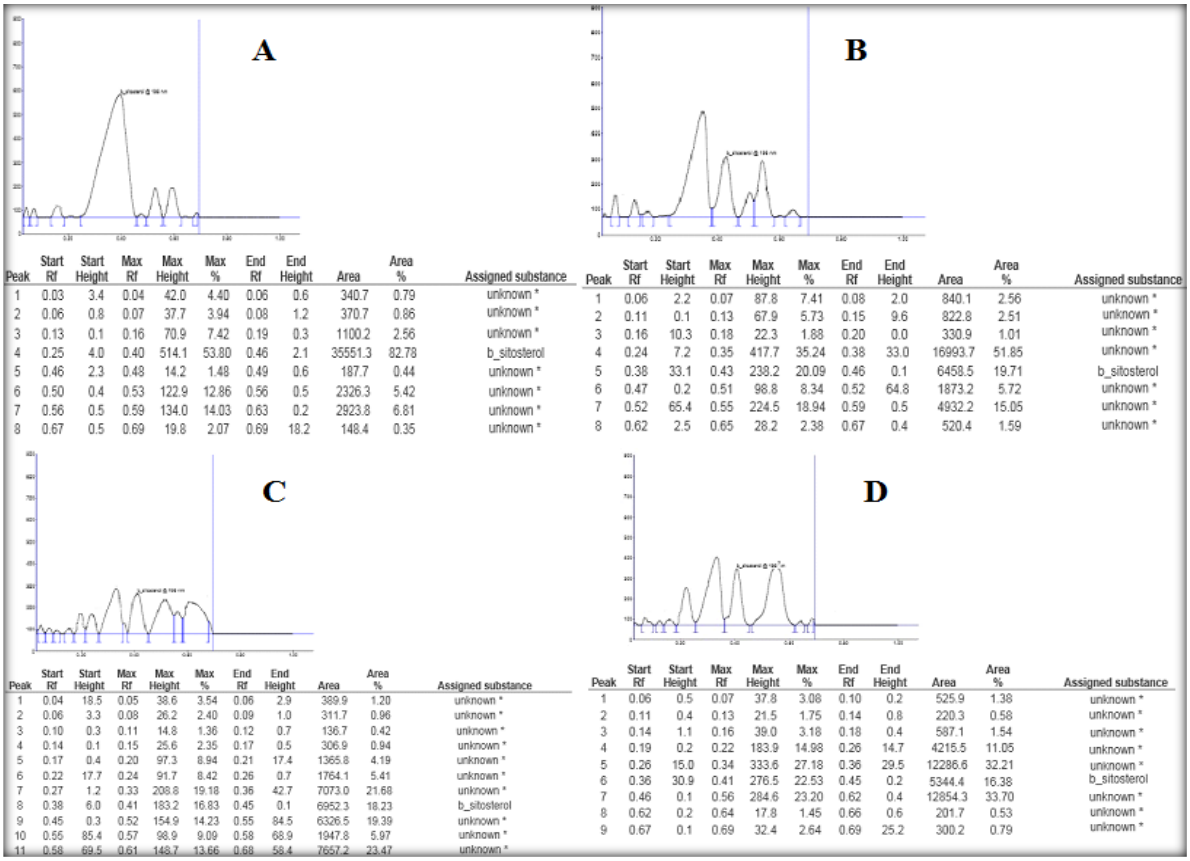
C

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.04	12.5	0.05	38.0	2.88	0.06	0.2	335.3	0.88	unknown *
2	0.06	1.0	0.08	38.3	2.91	0.09	1.6	438.7	1.15	unknown *
3	0.10	1.2	0.11	19.1	1.45	0.13	0.5	203.5	0.53	unknown *
4	0.14	0.4	0.15	23.4	1.78	0.17	0.1	253.4	0.66	unknown *
5	0.17	0.2	0.20	126.9	9.62	0.22	23.4	1775.6	4.64	unknown *
6	0.22	24.2	0.24	75.8	5.75	0.26	0.1	1327.9	3.47	unknown *
7	0.27	1.5	0.32	260.2	19.74	0.38	0.2	9023.7	23.59	unknown *
8	0.38	1.4	0.41	234.2	17.76	0.45	0.5	5668.2	18.13	b_sitosterol
9	0.46	0.1	0.52	192.0	14.56	0.55	47.4	6950.1	18.17	unknown *
10	0.56	47.6	0.57	65.2	4.94	0.59	26.2	1214.6	3.18	unknown *
11	0.59	28.1	0.63	245.4	18.61	0.69	47.6	11058.5	28.91	unknown *

D

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.13	0.0	0.15	16.8	1.93	0.17	1.4	294.8	1.24	unknown *
2	0.19	2.3	0.21	13.7	1.58	0.23	4.3	247.9	1.04	unknown *
3	0.24	4.4	0.31	245.4	28.28	0.34	27.4	7878.5	33.14	unknown *
4	0.34	27.9	0.35	31.2	3.60	0.36	19.6	417.5	1.76	unknown *
5	0.36	19.7	0.40	189.0	21.78	0.44	0.0	4311.3	14.82	b_sitosterol
6	0.45	0.9	0.51	78.0	8.99	0.54	26.3	3205.3	13.48	unknown *
7	0.54	27.0	0.58	149.4	17.22	0.61	35.4	4098.9	17.24	unknown *
8	0.61	36.0	0.63	51.5	5.94	0.64	46.5	934.1	3.93	unknown *
9	0.64	46.6	0.66	92.6	10.68	0.69	39.9	2386.4	10.04	unknown *

Figure (4): HPTLC chromatogram of petroleum ether fraction extracted from four parts of Awsaj plant by conventional (Soxhlet) method. A= Roots, B= Fruits, C= Leaves, D= stems.



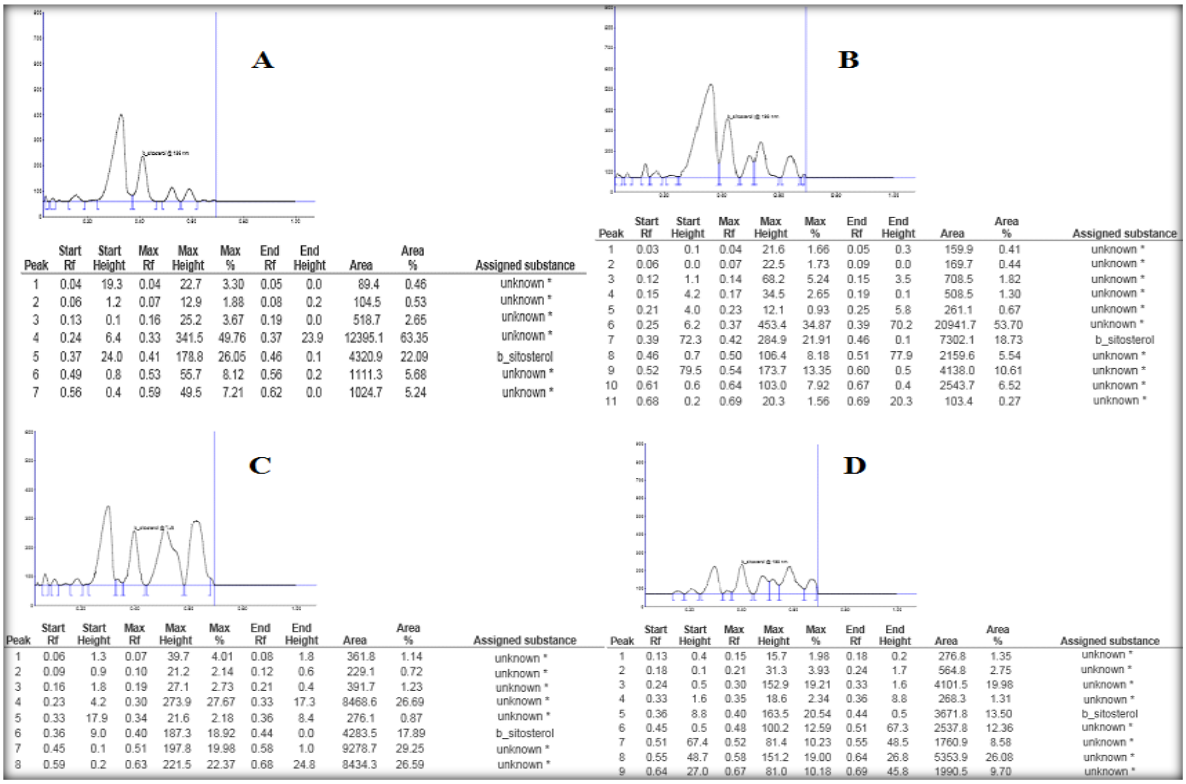
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	3.4	0.04	42.0	4.40	0.06	0.6	340.7	0.79	unknown *
2	0.06	0.8	0.07	37.7	3.94	0.08	1.2	370.7	0.86	unknown *
3	0.13	0.1	0.16	70.9	7.42	0.19	0.3	1100.2	2.56	unknown *
4	0.25	4.0	0.40	514.1	53.80	0.46	2.1	35551.3	82.78	b_sitosterol
5	0.46	2.3	0.48	14.2	1.48	0.49	0.6	187.7	0.44	unknown *
6	0.50	0.4	0.53	122.9	12.86	0.56	0.5	2326.3	5.42	unknown *
7	0.56	0.5	0.59	134.0	14.03	0.63	0.2	2923.8	6.81	unknown *
8	0.67	0.5	0.69	19.8	2.07	0.69	18.2	148.4	0.35	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.06	2.2	0.07	87.8	7.41	0.08	2.0	840.1	2.56	unknown *
2	0.11	0.1	0.13	67.9	5.73	0.15	9.6	822.8	2.51	unknown *
3	0.16	10.3	0.18	22.3	1.88	0.20	0.0	330.9	1.01	unknown *
4	0.24	7.2	0.35	417.7	35.24	0.38	33.0	16993.7	51.85	unknown *
5	0.38	33.1	0.43	238.2	20.09	0.46	0.1	6458.5	19.71	b_sitosterol
6	0.47	0.2	0.51	98.8	8.34	0.52	64.8	1873.2	5.72	unknown *
7	0.52	65.4	0.55	224.5	18.94	0.59	0.5	4932.2	15.05	unknown *
8	0.62	2.5	0.65	28.2	2.38	0.67	0.4	520.4	1.59	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.04	18.5	0.05	38.6	3.54	0.06	2.9	389.9	1.20	unknown *
2	0.06	3.3	0.08	26.2	2.40	0.09	1.0	311.7	0.96	unknown *
3	0.10	0.3	0.11	14.8	1.36	0.12	0.7	136.7	0.42	unknown *
4	0.14	0.1	0.15	25.6	2.35	0.17	0.5	306.9	0.94	unknown *
5	0.17	0.4	0.20	97.3	8.94	0.21	17.4	1365.8	4.19	unknown *
6	0.22	17.7	0.24	91.7	8.42	0.26	0.7	1764.1	5.41	unknown *
7	0.27	1.2	0.33	208.8	19.18	0.36	42.7	7073.0	21.68	unknown *
8	0.38	6.0	0.41	183.2	16.83	0.45	0.1	6952.3	18.23	b_sitosterol
9	0.45	0.3	0.52	154.9	14.23	0.55	84.5	6326.5	19.39	unknown *
10	0.55	85.4	0.57	98.9	9.09	0.58	68.9	1947.8	5.97	unknown *
11	0.58	69.5	0.61	148.7	13.66	0.68	58.4	7657.2	23.47	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.06	0.5	0.07	37.8	3.08	0.10	0.2	525.9	1.38	unknown *
2	0.11	0.4	0.13	21.5	1.75	0.14	0.8	220.3	0.58	unknown *
3	0.14	1.1	0.16	39.0	3.18	0.18	0.4	587.1	1.54	unknown *
4	0.19	0.2	0.22	183.9	14.98	0.26	14.7	4215.5	11.05	unknown *
5	0.26	15.0	0.34	333.6	27.18	0.36	29.5	12286.6	32.21	unknown *
6	0.36	30.9	0.41	276.5	22.53	0.45	0.2	5344.4	16.38	b_sitosterol
7	0.46	0.1	0.56	284.6	23.20	0.62	0.4	12854.3	33.70	unknown *
8	0.62	0.2	0.64	17.8	1.45	0.66	0.6	201.7	0.53	unknown *
9	0.67	0.1	0.69	32.4	2.64	0.69	25.2	300.2	0.79	unknown *

Figure (5): HPTLC chromatogram of petroleum ether fraction extracted from four parts of Awsaj plant by UAE probe method. A= Roots, B= Fruits, C= Leaves, D= stems.



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.04	19.3	0.04	22.7	3.30	0.05	0.0	89.4	0.46	unknown *
2	0.06	1.2	0.07	12.9	1.88	0.08	0.2	104.5	0.53	unknown *
3	0.13	0.1	0.16	25.2	3.67	0.19	0.0	518.7	2.65	unknown *
4	0.24	6.4	0.33	341.5	49.76	0.37	23.9	12395.1	63.35	unknown *
5	0.37	24.0	0.41	178.8	26.05	0.46	0.1	4320.9	22.09	b_sitosterol
6	0.49	0.8	0.53	55.7	8.12	0.56	0.2	1111.3	5.68	unknown *
7	0.56	0.4	0.59	49.5	7.21	0.62	0.0	1024.7	5.24	unknown *

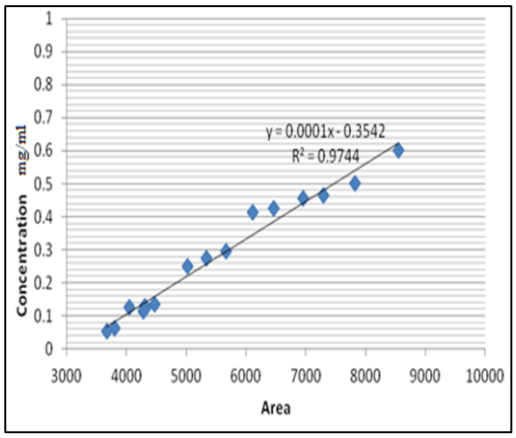
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.1	0.04	21.6	1.66	0.05	0.3	159.9	0.41	unknown *
2	0.06	0.0	0.07	22.5	1.73	0.09	0.0	169.7	0.44	unknown *
3	0.12	1.1	0.14	68.2	5.24	0.15	3.5	708.5	1.82	unknown *
4	0.15	4.2	0.17	34.5	2.65	0.19	0.1	508.5	1.30	unknown *
5	0.21	4.0	0.23	12.1	0.93	0.25	5.8	261.1	0.67	unknown *
6	0.25	6.2	0.37	453.4	34.87	0.39	70.2	20941.7	53.70	unknown *
7	0.39	72.3	0.42	284.9	21.91	0.46	0.1	7302.1	18.73	b_sitosterol
8	0.46	0.7	0.50	106.4	8.18	0.51	77.9	2159.6	5.54	unknown *
9	0.52	79.5	0.54	173.7	13.35	0.60	0.5	4138.0	10.61	unknown *
10	0.61	0.6	0.64	103.0	7.92	0.67	0.4	2543.7	6.52	unknown *
11	0.68	0.2	0.69	20.3	1.56	0.69	20.3	103.4	0.27	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.06	1.3	0.07	39.7	4.01	0.08	1.8	361.8	1.14	unknown *
2	0.09	0.9	0.10	21.2	2.14	0.12	0.6	229.1	0.72	unknown *
3	0.16	1.8	0.19	27.1	2.73	0.21	0.4	391.7	1.23	unknown *
4	0.23	4.2	0.30	273.9	27.67	0.33	17.3	8468.6	26.69	unknown *
5	0.33	17.9	0.34	21.6	2.18	0.36	8.4	276.1	0.87	unknown *
6	0.36	9.0	0.40	187.3	18.92	0.44	0.0	4283.5	17.89	b_sitosterol
7	0.45	0.1	0.51	197.8	19.98	0.58	1.0	9278.7	29.25	unknown *
8	0.59	0.2	0.63	221.5	22.37	0.68	24.8	8434.3	26.59	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.13	0.4	0.15	15.7	1.98	0.18	0.2	276.8	1.35	unknown *
2	0.18	0.1	0.21	31.3	3.93	0.24	1.7	564.8	2.75	unknown *
3	0.24	0.5	0.30	152.9	19.21	0.33	1.6	4101.5	19.98	unknown *
4	0.33	1.6	0.35	18.6	2.34	0.36	8.8	268.3	1.31	unknown *
5	0.36	8.8	0.40	163.5	20.54	0.44	0.5	3671.8	13.50	b_sitosterol
6	0.45	0.5	0.48	100.2	12.59	0.51	67.3	2537.8	12.36	unknown *
7	0.51	67.4	0.52	81.4	10.23	0.55	48.5	1760.9	8.58	unknown *
8	0.55	48.7	0.58	151.2	19.00	0.64	26.8	5353.9	26.08	unknown *
9	0.64	27.0	0.67	81.0	10.18	0.69	45.8	1990.5	9.70	unknown *

Figure (6): HPTLC chromatogram of petroleum ether fraction extracted from four parts of Awsaj plant by UAE bath method. A= Roots, B= Fruits, C= Leaves, D= stems.

For quantification measurements, the calibration curve were plotted using area under the curve (AUC) versus four concentration levels of ß-sitosterol standard. A straight line equation were obtained from which the concentration of the analyte was calculated in each part of Awsaj plant as shown in figure 7.



Figure(7): Calibration curve of β- sitosterol standard on HPTLC

Quantitative concentration of β-sitosterol in petroleum ether fraction revealed that roots contain the higher concentration of ß-sitosterol followed by fruits, leaves and the least was stems. The high concentration of β-sitosterol in roots of Iraqi wild grown Awsaj may be attributed to the availability of biochemical factors which are considered essential elements for the formation of building back of this secondary metabolite. The probe UAE gives the higher concentration of ß-sitosterol followed by Soxhlet and last by bath UAE, as shown in (table 2) and (figure 8).

Table(2): Quantitative analysis of β-sitosterol in each part of Awsaj by HPTLC

Part of plant	Concentration mg/ml by Soxhlet	Concentration mg/ml by probe UAE	Concentration mg/ml by bath UAE
Roots	0.465	0.610	0.412
Fruits	0.295	0.485	0.122
Leaves	0.135	0.405	0.113
Stems	0.120	0.275	0.0534

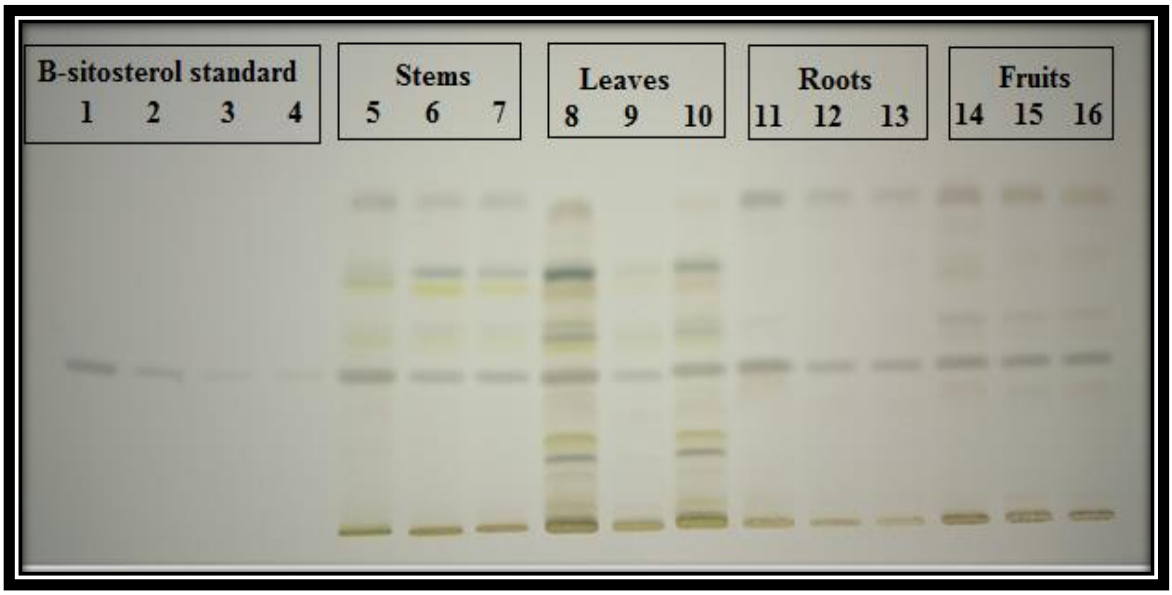


Figure (8): HPTLC, track 1-4= β-sitosterol standard, track 5-16= petroleum ether fraction obtained by probe UAE, bath UAE and Soxhlet methods respectively for each part of Awsaj.

Isolation of β-sitosterol by preparative HPLC

According to HPTLC results; isolation and purification of ß-sitosterol from the root parts of Iraqi Awsaj plant extracted by probe UAE will be done by preparative HPLC which is considered now the most powerful and versatile method for purification tasks in pharmaceutical industries. PHPLC chromatogram showed twelve peaks which represent twelve different compounds according to their retention time, one of them (β-sitosterol) at retention time of 1.142, is a major peak which was identified by comparison with standard β-sitosterol at retention time of 1.123. The major peak was collected by fractions collector after monitoring it according to the time (time from

beginning of peak appearance until start to disappearing of the peak) figure 9 and 10, then the sample obtained from PHPLC was dried over anhydrous sodium sulfate, weighted and subjected to different identification methods.

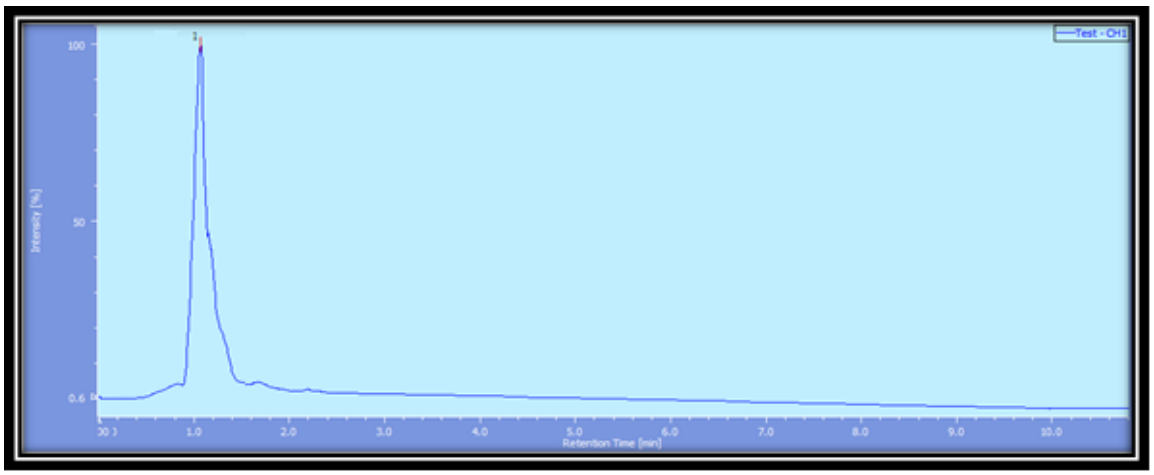
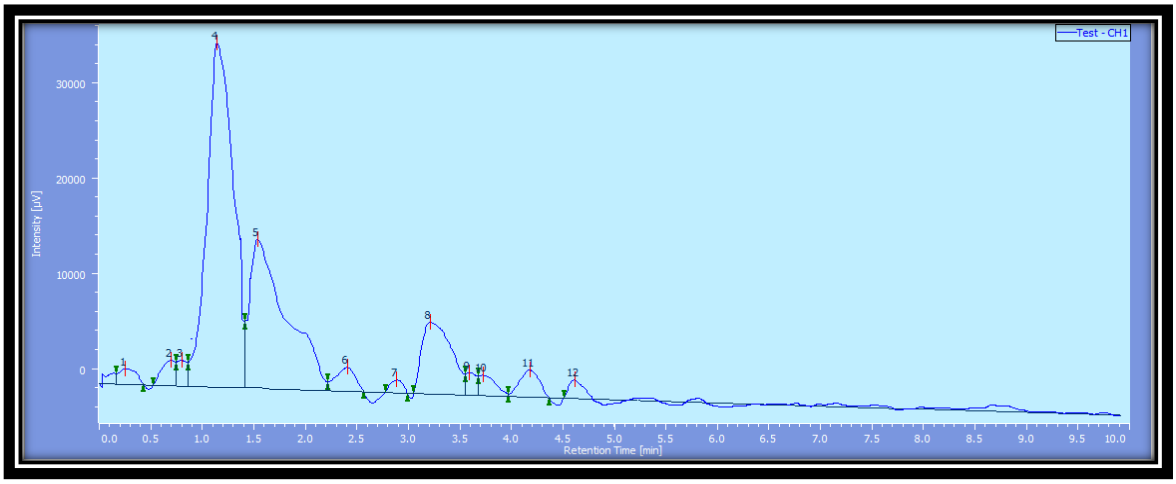


Figure (9): PHPLC chromatogram of standard β-sitosterol



Figure(10): PHPLC chromatogram of roots petroleum ether fraction

Characterization of the isolated ß-sitosterol

1- Thin layer chromatography (TLC):

The isolated compound appeared as a single spot having the same color and R_f value as that of standard β-sitosterol as shown in (figure 11).

2- Measuring melting point:

The isolated compound had a sharp melting point 138-140 C°, compared to melting point 139-140 C° for the ß-sitosterol standard [23] .

3- Fourier transforms infrared (FT-IR) spectra:

FT-IR spectroscopy is most commonly used in phytochemical studies as a finger printing device, for comparing a natural with the synthetic reference standard, so IR spectra of isolated compound gave identical results which was compared with standard β-sitosterol as shown in figure 12 and 13, and the characteristic IR absorption bands of isolated compound are listed in (table 3) [24] .

Figure (11): TLC Chromatogram of isolated compound (ISO) with β-sitosterol standard (ST) on silica gel GF 254, developing in toluene – ethyl acetate - chloroform 5:1:4 and detection by spraying with 5% H2SO4 + heating at 110 Cº.

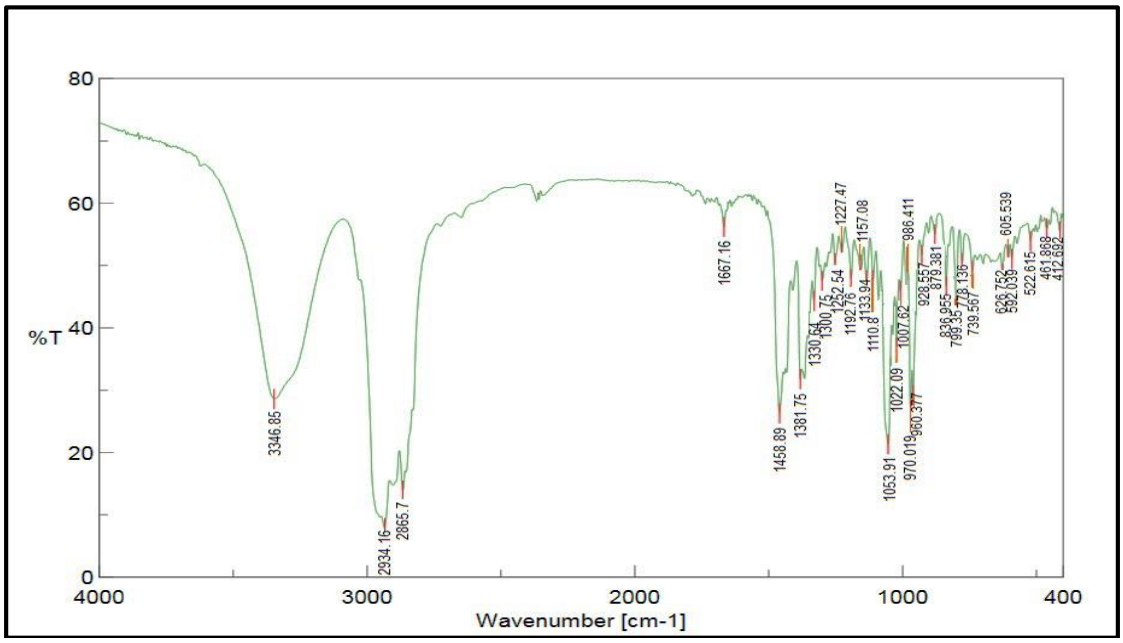


Figure (12): IR Spectrum of standard β-sitosterol

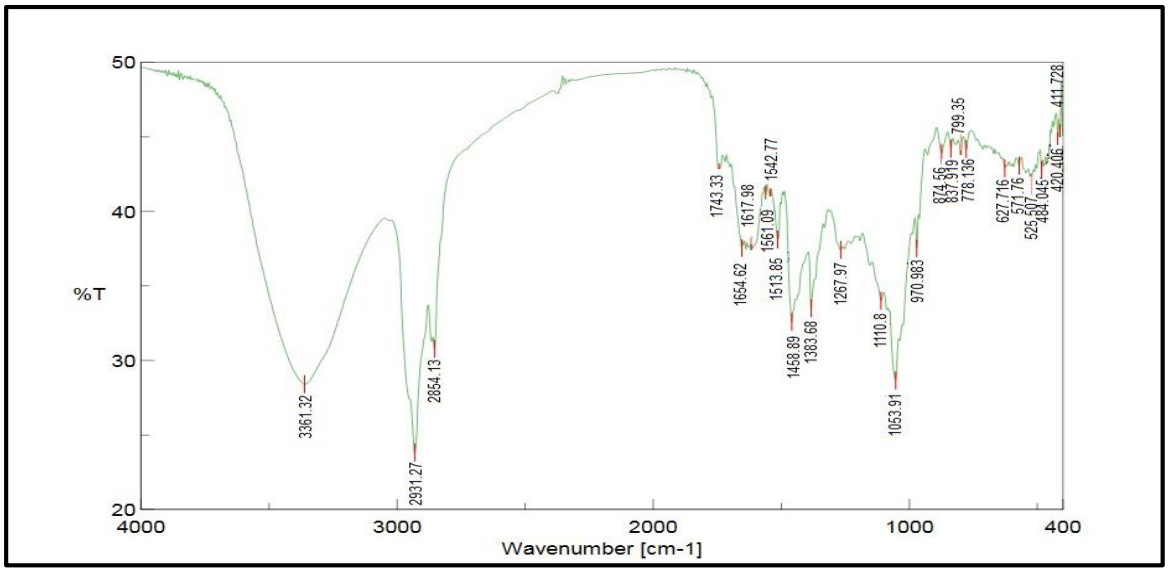


Figure (13): IR Spectrum of isolated β-sitosterol

Table(3): Characteristic infra-red frequencies of isolated ß-sitosterol compound

Phytosterol compound	Approximated position of characteristic bands (cm^{-1})
β-sitosterol	3361.32 (free O-H stretching band), 2931.27 and 2854.13 (aliphatic C-H stretching), 1654.62 (C=C absorption peak), 1458.89 (C-H bending /-CH2), 1053.91 and 874.56 (cycloalkane)

4- Preparative HPLC

PHPLC was used again for characterization and identification by comparing the retention time of the isolated (1.117) with that of standard β-sitosterol (1.123) as shown in (figure 14).

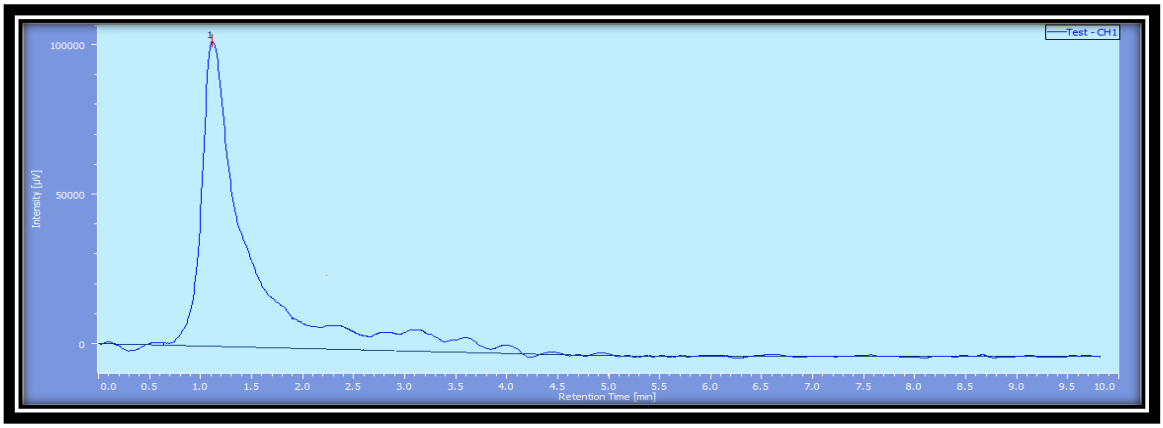


Figure (14): PHPLC chromatogram of isolated ß-sitosterol

Conclusion

From the above findings, the utilization of probe UAE has proved to be much simpler and more effective than the conventional Soxhlet and bath UAE extraction methods for obtaining ß-sitosterol from fruits, leaves, stems and roots of Iraqi Awsaj plant. HPTLC and comparison of standard facilitated the identification and quantification of B-sitosterol present in petroleum ether fraction of the four parts of Awsaj plant. ß-sitosterol was isolated from petroleum ether fraction of root parts of Awsaj and characterization was carried out by measuring of melting point follow by running two chromatographic procedures (TLC and PHPLC) and finally spectral characteristic (FTIR).

The current research also recommends using the other bioactive Phytosterols in order to confirm the presence of them in Iraqi Awsaj plant.

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