Isolation and Characterization of Triterpenoid Saponin Hederacoside C. Present in the Leaves of *Hedera helix* L. Cultivated in Iraq

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Abstract

Hedera helix L. plant belongs to the family Araliaceae that provide a host of bioactive compounds (mainly saponins) of important biological activities, like spasmolytic, secretolytic, anti-inflammatory, and antibacterial activities. Literature survey revealed that there was no previously study concerning *H. helix* L. which is cultivated in Iraq, so we decided to carry out this study which include extraction, isolation, purification and identification of biologically important triterpenoid saponin hederacoside C from leaves of *H. helix* L. Extraction of hederacoside C was carried out using two methods; in the first method maceration was done with methanol 99.8% and in the second method soxhlet extraction with ethanol 99.8%, was followed, then fractionation using column chromatography. Preliminary identification of this saponin hederacoside C was done using thin layer chromatography (TLC) where different solvent systems had been tried. Liebermann-Burchard reagent where used for detection. The most suitable extraction method was fully described in this study. The characterization of the isolated hederacoside C was carried out using melting point (M.P.), thin layer chromatography (TLC), FT-IR, and high performance liquid chromatography (HPLC).

Keywords: Hedera helix L., Hederacoside C, Column chromatography.

فصل وتوصيف الترايبنويد الصابوني (الهيدراكوسايد سي) الموجود في اوراق نبات اللبلاب الكبير المستزرع في العراق.

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الخلاصة:

يعد نبات اللبلاب الكبير من عائلة (Araliaceae) وهو يحتوي على الكثير من المركبات الكيميائية و بشكل رئيسي الكلايكوسيدات الصابونية ثلاثية الترايبنويد (triterpenoid saponin glycosides) ذات الفعالية الاحيائية كمضاد للتقلصات ومضاد للالتهابات. ونظراً لعدم وجود دراسات حول نبات اللبلاب الكبير في العراق, لذا اصبح من الاهمية دراسة هذا النبات المستزرع في العراق. في هذه الدراسة تم استخلاص وكشف وفصل وتنقية الكلايكوسيد الصابوني الترايبنويد (hederacoside C) من اوراق نبات اللبلاب الكبير (H. helix L.) حيث تم الاستخلاص باستخدام طريقتين: الطريقة الاولى باستخدام النقيع في الكحول المثيلي 99.8% والطريقة الثانية باستخدام جهاز السوكسليت ومذيب الكحول الاثيلي ٩٩.٨%,ومن ثم التجزئة باستخدام كروماتوكرافيا العمود (column chromatography). تم الكشف عن الكلايكوسيد الصابوني (hederacoside C) بأستخدام تقنية كروماتوغراقيا الطبقة الرقيقة, باستخدام مذيبات مختلفة كوسيط ناقل و الكشف عنها بواسطة كاشف (Liebermann-Burchard), وبعدها تمت عملية الفصل و التنقية. كما تم في هذه الدراسة اختيار الطريقة المناسبة للاستخلاص والفصل و التنقية و شرحها بالتفصيل. كذلك تم استخدام مجموعة من التقنيات للتحقق من المركب المفصول (hederacoside C) و درجة نقاوتها والتي شملت: (قياس درجة الانصهار للمركب المفصول وكروماتوغرافيا الطبقة الرقيقة ومطياف الاشعة تحت الحمراء و كذلك تقنية كروماتوغرافيا الاداء العالي السائلة) والتي تطابقت نتائجها مع نتائج (hederacoside C) القياسي.

الكلمات المفتاحية: نبات اللبلاب الكبير, الهيدراكوسايد سي, كروماتوكرافيا العمود.

Introduction

Hedera genus is one of the 55 genera in Araliaceae family[1]. Members of the genus are highly valued as ornamentals, and are commonly used in the landscape as well as indoors. Recently examined species delimitations within *Hedera* genus recognize 12 species, three subspecies, and one variety. *Hedera helix* L[2].(English ivy, Common ivy) is an evergreen dioecious woody liana, one of the 15 species of the genus *Hedera*. The whole plant leaves are coriaceous, 4-10 cm long and wide, cordate at the base.

The lamina is palmately 3-5 lobed. The upper surface is dark green with a paler, radiate venation while the lower surface is more grenish-green and the venation is distinctly raised [3]. *H. helix* L. naturally grows in the Western, Central, and Southern Europe but has also been introduced to North America and Asia[4]. Saponins can be classified into groups based on the nature of the aglycone skeleton. The first group consists of the steroidal saponins, which are almost exclusively present in the monocotylednous angiosperms.

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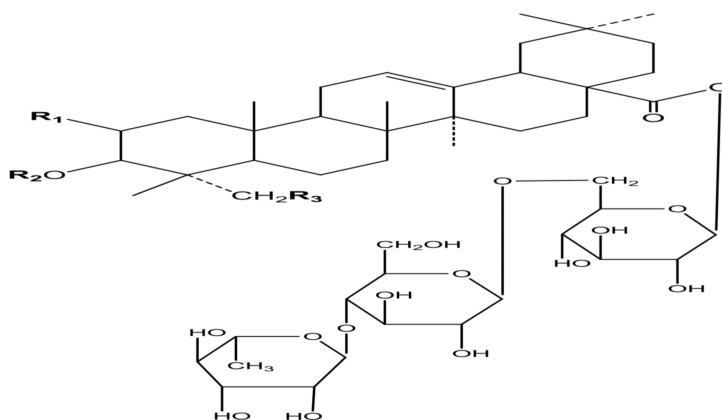
The second group consists of the triterpenoid saponins, which are most common and occur mainly in the dicotyledonous angiosperms[5]. The biologically active compounds responsible for the medicinal use of the plant are triterpenoid saponins (2.5-6%): the bidesmosidic glycosides of hederagenin and oleanolic acid (hederacoside C,B,D,E,F,G,H,I) and the monodesmoside α-hederin. Other groups of the identified compounds are represented by phenolics (flavonoids, anthocyanins, coumarins, and phenolic acids), amino acids, steroids, vitamins, volatile and fixed oils, β-lectins, and polyacetylenes. The pharmacological activities of *Hedera helix* L. are: spasmolytic, secretolytic, anti-inflammatory, antimicrobial, antiviral, antifungal, protozoidal, hepatoprotective, antioxidant, hypoglycaemic, cytotoxic, antimutagenic, and anti-hyaluronidase activity[3].

Figure 1: Photo of *H. helix* L. [6]

Table(1): Chemical structure of the major saponin glycosides of *H. helix* L. leaves[7,8]



Saponin glycoside	R1	R2	R3
Hederasaponin B	H	[α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl	H
Hederasaponin C	H	[α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl	OH
Hederasaponin D	H	α-L-arabinopyranosyl	OH
Hederasaponin E	OH	α-L-arabinopyranosyl	OH
Hederasaponin F	H	β-sulfate	H
Hederasaponin G	H	[β-D-glucopyranosyl-(1→2)-α-L-arabinopyranosyl]	OH
Hederasaponin I	H	β-D-glucuronopyranosl	OH

Approved therapeutic uses of *H. helix* L. extract:[9]

- Acute catarrh (inflammation) of the respiratory tract accompanied by coughing.
- Symptomatic treatment of chronic inflammatory bronchial diseases

Materials and methods

Plant materials

The whole plant *H. helix* L. was collected during November 2012 from Zayona district of Baghdad, identified and authenticated by prof. Dr. Ali Al-Musawi, university of Baghdad. The leaves were cut from the whole plant, cleaned gently, and dried in oven at 40 °C , then coarsely powdered by mechanical grinder and weighed.

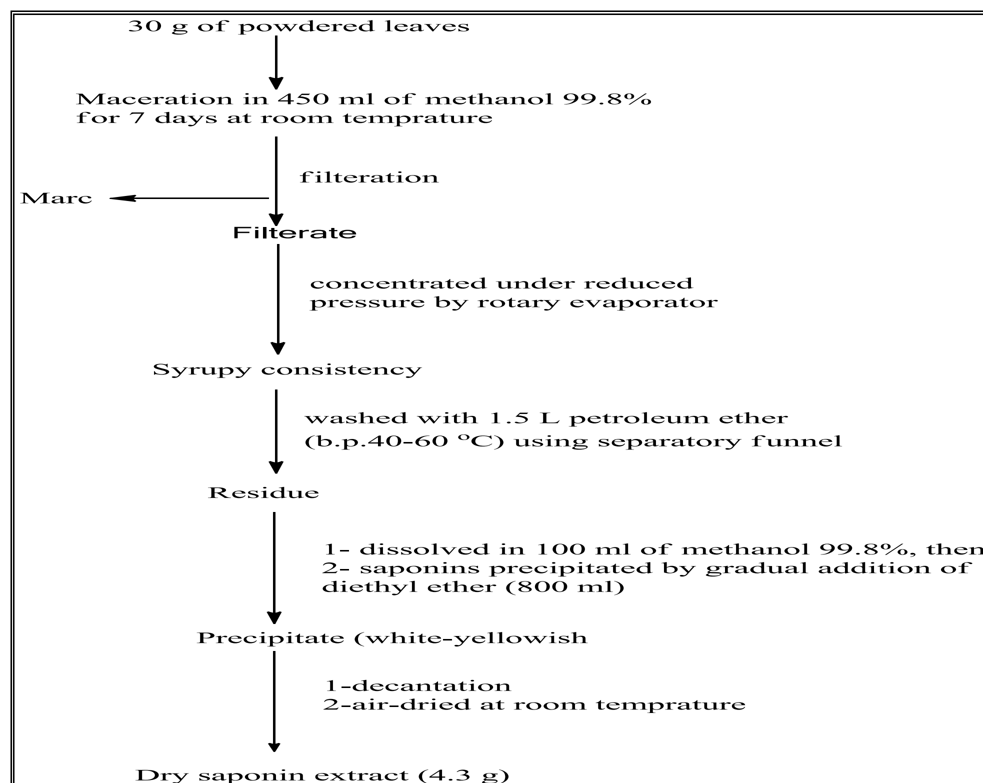
Extraction methods of triterpenoid saponins from H. helix L. leaves:

Extraction method No.1 [10]

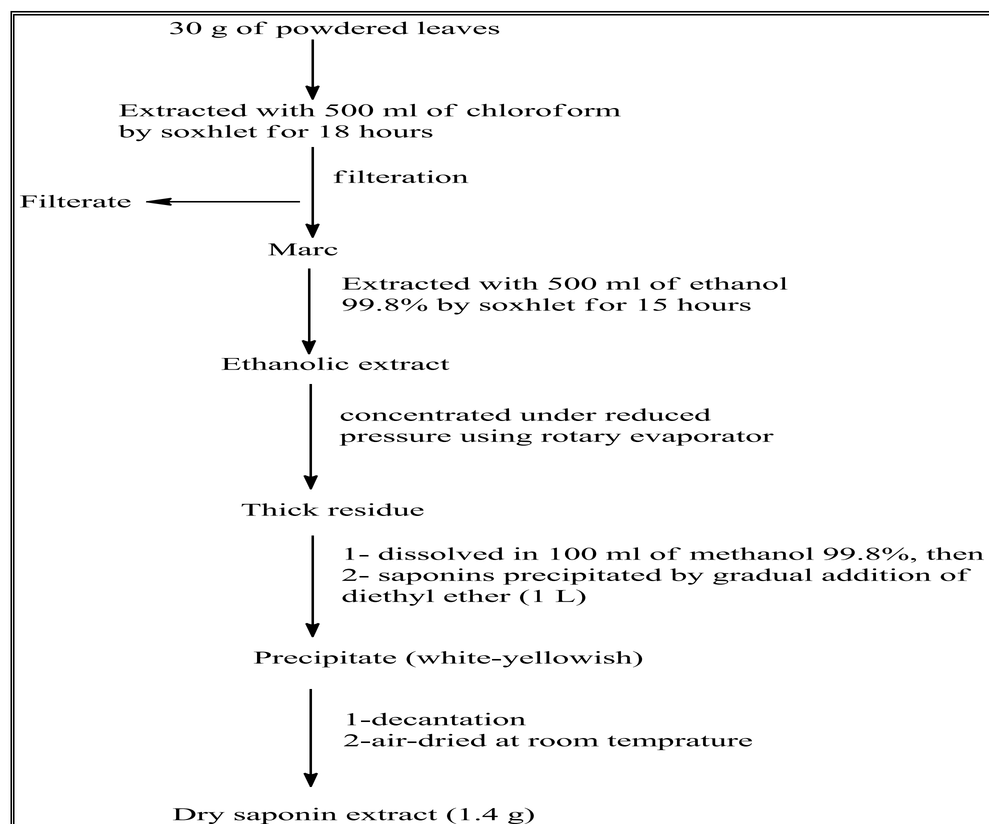
Thirty grams of the powdered leaves of *H. helix* L. were extracted by maceration in (450 ml) of methanol 99.8% for seven days at room temperature and then filtered. The filterate was evaporated at reduced pressure in the rotary evaporator to a thick residue. This residue was repeatedly washed with petroleum ether (b.p. 40-60 °C) to remove the chlorophyll and fatty materials.The process was continued till there were no traces of colouring matter in the petroleum ether. A thicky residue obtained which redissolved in (100 ml) of methanol 99.8%. To this methanolic solution diethyl ether was added. A white-yellowish precipitate of saponin was formed. The addition of diethyl ether was continued, until no further precipitate was formed. The precipitate was recovered by decantation ,then air dried at room temperature to yield the crude extract (4.3 g). Scheme (1) shows the general procedure for this extraction method.

Extraction method No.2 [10]

Thirty grams of powdered leaves of *H. helix* L. were first extracted with (500 ml) of chloroform in a soxhlet apparatus for 18 hours. The residue left was next extracted with (500 ml) of ethanol 99.8% for 15 hours in soxhlet apparatus. The ethanolic extract was evaporated under reduced pressure to a residue which was next dissolved in (100 ml) of methanol 99.8%. To this methanolic solution diethyl ether was added. A white-yellowish precipitate of saponin was formed. The addition of diethyl ether was continued, until no further precipitate was formed. The precipitate was recovered by decantation, then air dried at room temprature to yield the dry crude extract (1.4 g). Scheme (2) shows the general procedure for this extraction method.



Scheme (1): The general schematic procedure for the extraction method No.1



Scheme (2): The general schematic procedure for the extraction method No.2

Preliminary identification of the triterpenoid saponin glycosides

Identification of the triterpenoid saponin glycosides were carried out by thin layer chromatography (TLC) using ready made aluminum plates of silica gel GF254, and detection was done by Liebermann-Burchard reagent, using four different solvent systems.
Reference standard hederacoside C (ChromadexTM Company-USA).
Different developing solvent systems were:
S_1= chloroform : methanol : water (55:37:7)[8]
S_2= n-butanol : glacial acetic acid : water (60:15:15)[11]
S_3= chloroform : glacial acetic acid : methanol : water (60:32:12:8)[12]
S_4= n-butanol : glacial acetic acid : water (40:10:50)[13]

Reagent used for detection

Liebermann-Burchard reagent used for detection of triterpenoid saponin, and it is prepared by carefully adding 5 mL of acetic anhydride and 5 mL of concentrated sulfuric acid into 50 mL of absolute ethanol, while cooling in ice. Spray the developed plate and heat it at 100 ^{o}C for 5–10 minutes.[14]

Isolation and purification of hederacoside C

The dry crude extract obtained from extraction method No.1 of triterpenoid saponins was used for isolation and purification of hederacoside C, and performed as the following:

Fractionation by column chromatography

Two gram (2 g) of crude dry extract obtained from extraction method NO.1 was subjected to column chromatography using glass column (50 cm x 2.5 cm) packed with a slurry of silica gel (0.063-0.200 mm) in chloroform (wet method), in a ratio of 20 g of silica gel to each 1 g of the extract. A dry loading of the sample (crude extract) was used by dissolving it in small volume of methanol and adsorbing it on small amount of silica gel of the same grade used for packing the column, then dried, grinded and applied to the column in order to prevent clogging, since the sample is insoluble in the initial solvent chloroform. The column was eluted by gradient elution technique using chloroform: methanol with an increasing percentage of methanol from zero to 45% (the ratios of chloroform: methanol used were 100:0, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, and 55:45). The column developed by adding 100 ml of the eluent (chloroform : methanol), except for the ratios (60:40 and 55:45 of the eluent) the volumes of eluents were 200 ml and 100 ml, respectively then each 5 ml fractions were collected separately and monitored by TLC using developing solvent

system S_1. A total number of 130 fractions were obtained. Those consecutive fractions, which have the same number of spots with the same R_f values, were combined and evaporated to dryness to get the major fractions, F1, F2, F3 and F4, as shown in table (2).

Table (2): Major fractions obtained from column chromatography

Major fractions	No. of collections 5 ml each	No. of spots
F1	35-50	2
F2	51-78	3
F3	79-115	4
F4	116-130	4

Preparative TLC plates

The major fraction (F3) obtained by column chromatography was applied as a concentrated solution in a row of spots using capillary tube four times on each plate (the spots should dry before the next application). The thickness of silica gel on the plate was 0.75 mm. The solvent system (S_1) was placed in a glass tank (22.5 cm X 22 cm X 7 cm),and covered with a glass lid and allowed to stand for 45 minutes for saturation before use. The detection was done using Liebermann-burchard spray reagent in one side of the plate and by UV at 254 nm wave length. The target band was recovered by scraping off the adsorbent at the appropriate places on the developed plates, and collected in a beaker, mixed with chloroform: methanol (60:40), stirred with gentle heating, then filtered. After evaporation of the solvent, the obtained residue was subjected to co-TLC with the reference standard of hederacoside C using different S_1, S_2, S_3, and S_4 as solvent systems for identification and checking the purity of isolated compound.

Qualitative estimation of hederacoside C using HPLC technique

Qualitative estimation of hederacoside C was performed by using Waters/Ireland High Performance Liquid Chromatography (HPLC) in which identification was made by comparison of the retention time obtained at identical chromatographic conditions of the analyzed samples and authentic standard. The HPLC condition for hederacoside C is listed in the following table (3):

Table (3): HPLC condition for hederacoside C [15]

Mobile phase	Gradient: solvent A=0.1% glacial aetic acid in water, solvent B=0.1% glacial acetic acid in acetonitrile, isocratic 5% B for 5 minutes, then increasing to 95% B over 20 minutes.
Column	Phenomenex C_{18} 250 mm x 4.5mm, 5 µm particle size
Column temperature	Ambient
Flow rate	1 ml / min.
Injection volume	20 µL.
Injection concentration	2 mg /ml
Detection	UV Detector at λ 210 nm

Results

Extraction of triterpenoid saponins from the dried leaves of *H. helix* L. were done by two extraction methods and the method No.1 showed higher yield of dry crude extract and hederacoside C than method No.2, as shown in table (4). So the method No.1 was chosen for isolation and purification.

Table (4): Percentage of crude extracts obtained from extraction methods

Extraction method	% yield of crude extract
Method No.1	14.4 %
Method No.2	4.65 %

Identification of triterpenoid saponin (hederacoside C) by TLC

TLC of the major fraction (F3) obtained from fractionation by column chromatography of 2 g of crude extract from extraction method No.1, confirms the presence of hederacoside C in this major fraction in comparison with reference standard of hederacoside C, as represented in table (5) and figure (2).

Table (5): The R_f values of isolated hederacoside with its reference standard in different developing solvent systems using TLC technique:

Solvent system	S_1	S_2	S_3	S_4
R_f value of standard hederacoside C	0.72	0.44	0.35	0.5
R_f value of isolated hederacoside C	0.72	0.43	0.34	0.5

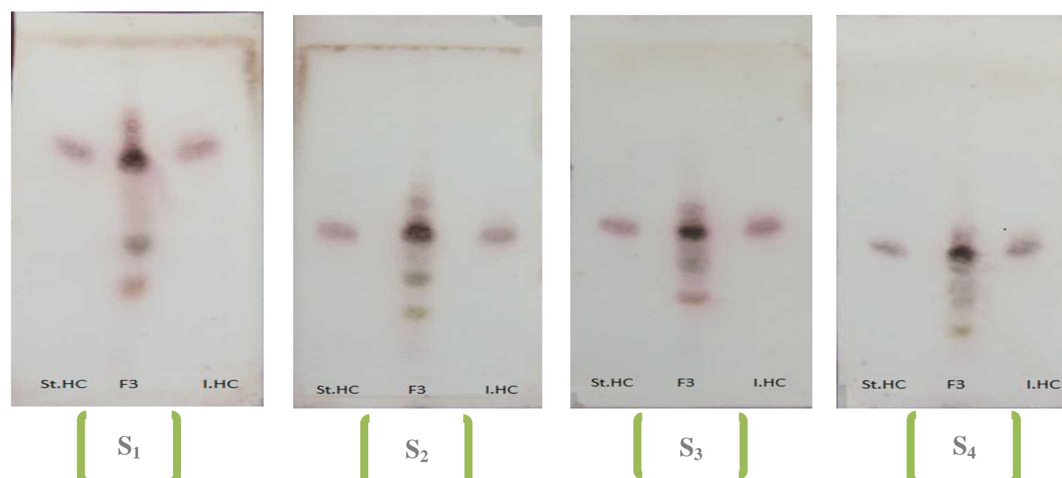


Figure 2: TLC of hederacoside C standard(St.HC), the major fraction (F3), and isolated hederacoside C (I.HC), using four solvent systems (S_1, S_2, S_3, S_4) as developing solvent systems. Detection by Liebermann-Burchard reagent.

Identification and characteriza-tion of the isolated hederacoside C

TLC

In analytical TLC using spiking technique in four different mobile phases, the isolated hederacoside C appears as a single spot having the same colour and R_f value as the reference standard of hederacoside C, as shown in figure (2).

Measuring melting points

The isolated compound was identified to be hederacoside C from its sharp melting point of (212-215 ^{o}C) compared to reference standard of hederacoside C melting point (214-215 ^{o}C).

FT-IR

The IR spectrum of isolated triterpenoid saponin hederacoside C was recorded as KBR disc which gave identical results as compared with reference standard of hederacoside C, as shown in table (6) and figure (3):

HPLC (High Performance Liquid Chromatography)

Qualitative and quantitative estimations of hederacoside C was done by using High performance liquid chromatography (HPLC) in which identifications were made by comparism of retention times obtained at identical chromatographic conditions of analyzed sample and authentic standard of hederacosid C, as shown in figure (4) and figure (5).

Table (6): The characteristic IR absorption bands in (cm^{-1}) of the isolated hederacoside C: [16]

Functional group	Hederacoside C standard	Isolated CM2 compound	Assignment
O-H	3495	3439	Broad O-H stretching band indicating hydrogen bonding
C-H	2962,2929,2875	2983,2937,2910	Asymmetric and symmetric stretching of CH3 and CH2
C=C	1660	1647	Stretching of C=C bond
C=O	1700	1697	C=O stretching of carbonyl group
C-H	1450,1380	1458,1365	Asymmetric and symmetric stretching of CH3 and CH2
O-H	1410	1435	O-H bending of alcohol
-C-O-C	1170	1122	Stretching of ether

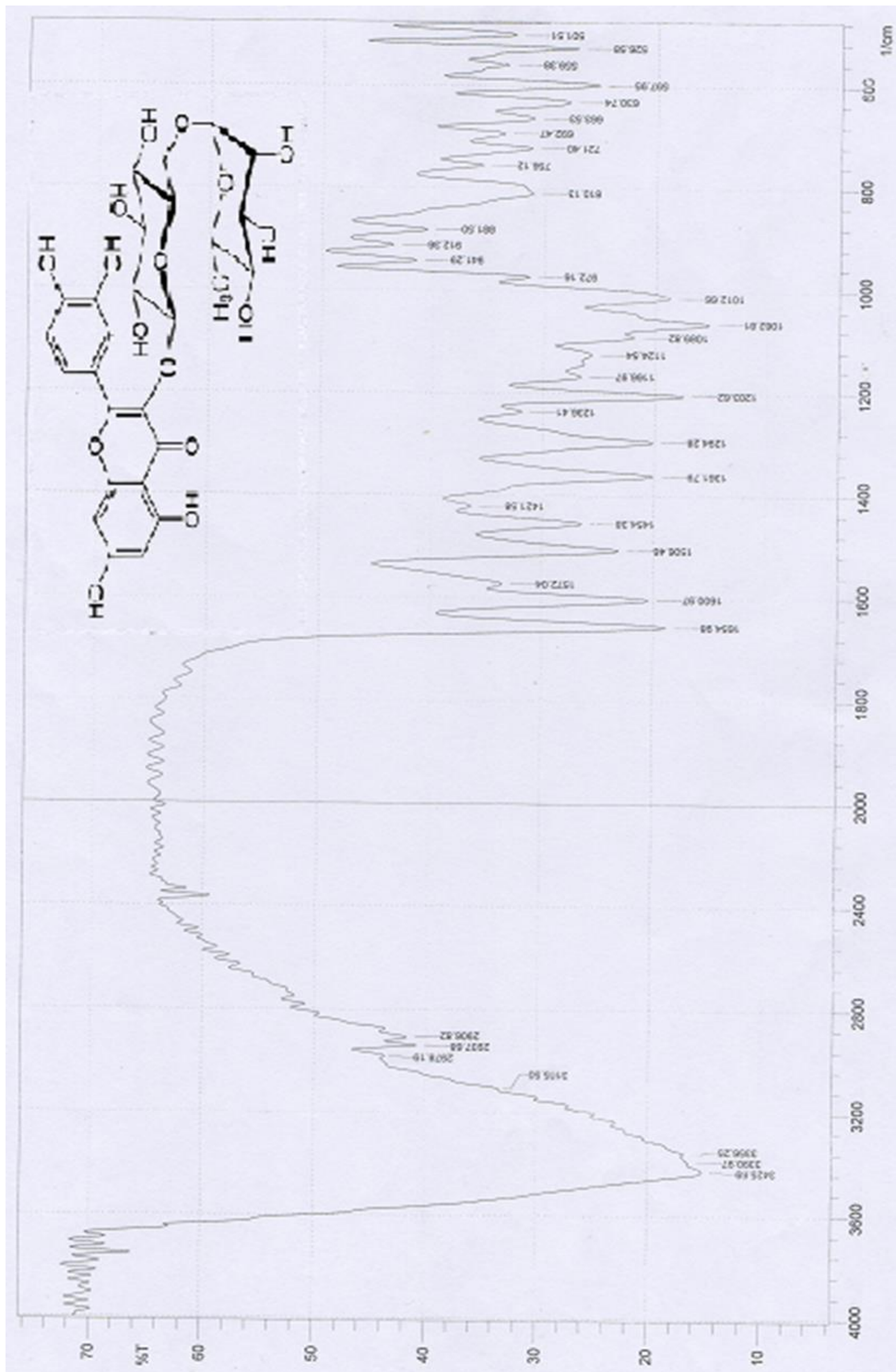


Figure 3: IR spectrum of the isolated hederacoside C

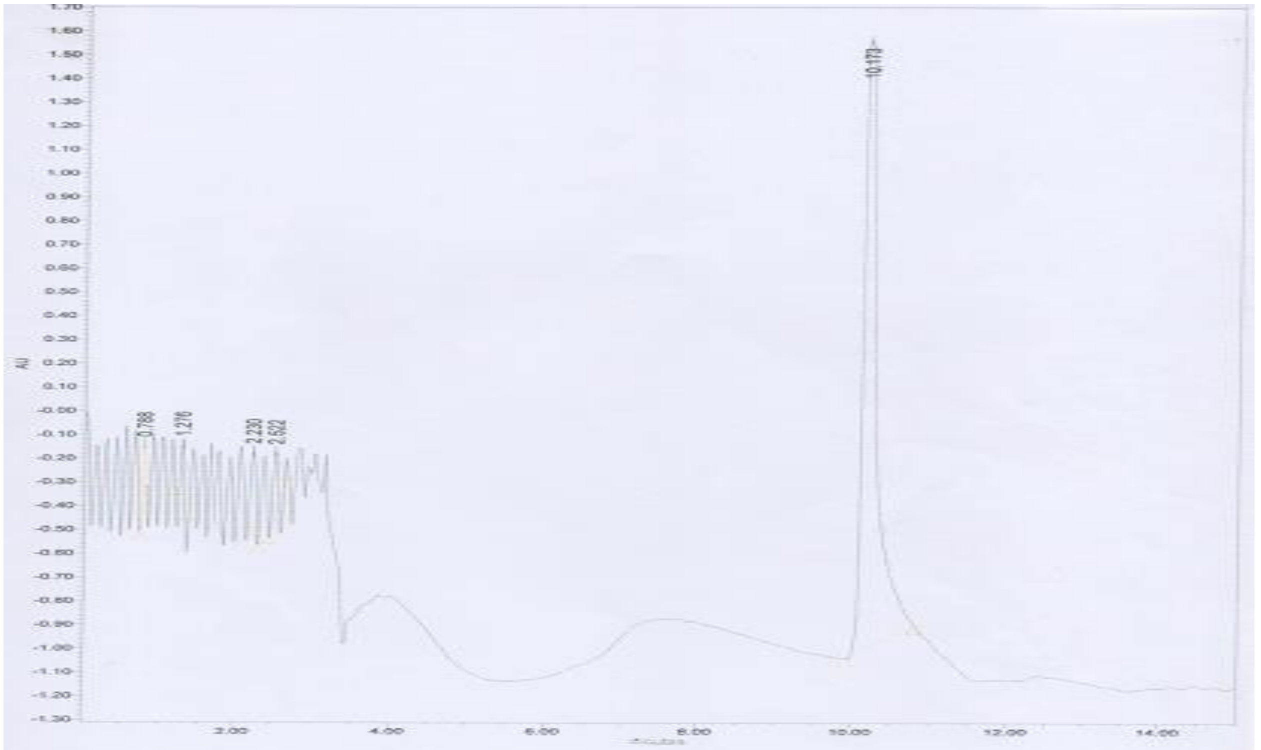


Figure 4: HPLC analysis of hederacoside C reference standard .

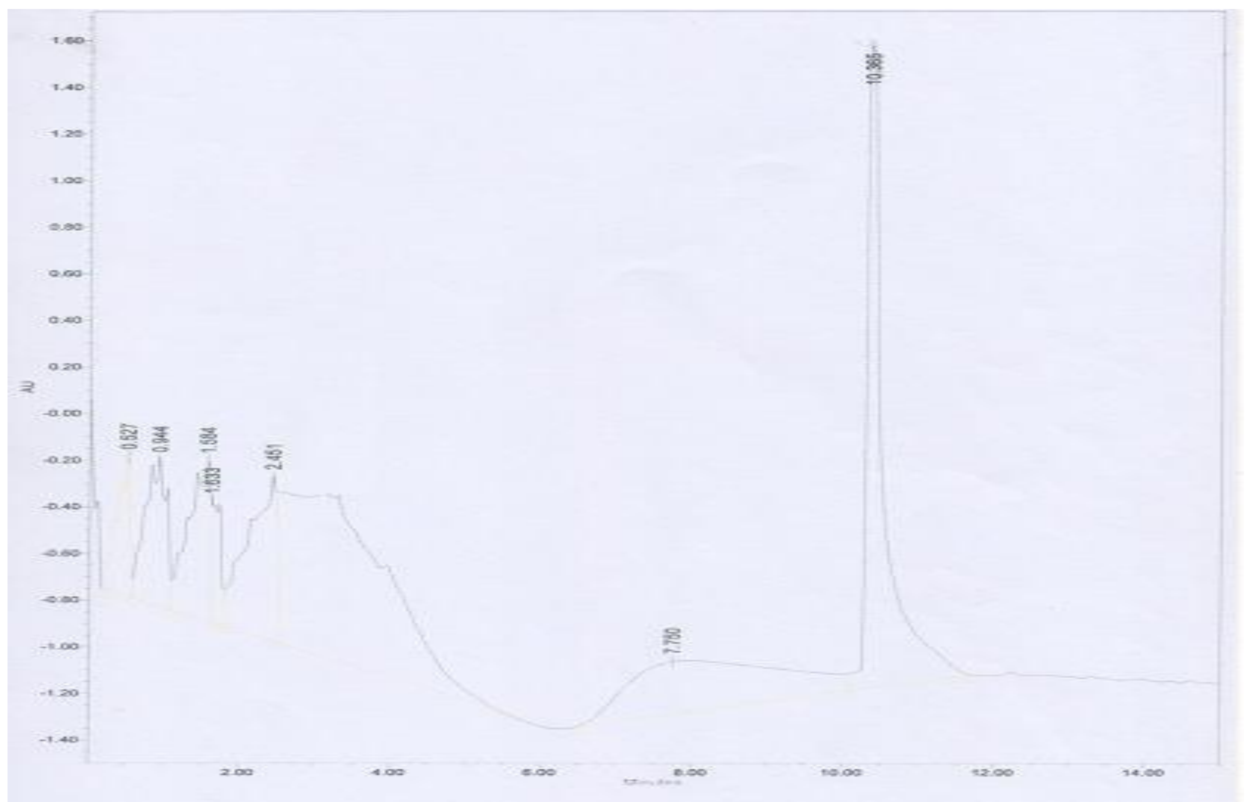


Figure 5: HPLC analysis of isolated hederacoside C

Conclusions

Phytochemical investigation of *H. helix* L. leaves, which cultivated in Iraq revealed the presence of important group of medicinal natural products belong to triterpenoid saponin glycosides. The crude extract fractionated by column chromatography, then hederacoside C isolated by preparative TLC. The isolated hederacoside C was by TLC, melting point, FT-IR, and HPLC analysis. This needs to perform other pharmacological studies to highlight its actions as therapeutic agent.

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