

Investigation of Some Phytochemical Compounds Found in *Anchusa strigosa* L. Grown Naturally in Iraq

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Abstract

Anchusa strigosa L.: perennial herb, with hairs especially on the leaves., flowers generally regular. Commonly named (Lisan Althour) in Iraq, from Boraginaceae family. The plant contain phenolic acids, flavonoids, alkaloids, sterols and terpenoids. Whole plant part defatted n-hexane for 24 hours. The defatted plant material extracted using absolute methanol by Soxhlet apparatus until complete exhaustion, the extract fractionated by solvents of different polarity: petroleum ether- chloroform - ethylacetate- and n-butanol respectively. The n-butanol fraction hydrolyzed with 5% HCl for 5 hours by reflux to break down the glycosidic linkage. Rosmarinic acid, caffeic acid, genistein and silybin were isolated from ethyl acetate fraction by preparative thin layer chromatography which identified by high performance liquid chromatography HPLC, Fourier transforms infrared (FTIR) spectra, thin layer chromatography TLC and melting point. Since the plant contain alkaloids so acid-base extraction was performed for crude extract resulting from maceration of the plant parts in methanol (cold method) to obtain the alkaloid that isolated by preparative thin layer chromatography and then identified by Fourier transforms infrared (FTIR) spectra and thin layer chromatography (TLC).

The aim of this research was to carry out a phytochemical study of this plant since no previous phytochemical investigation work had been done on this species in Iraq

Keywords: *Anchusa strigosa*, Rosmarinic acid, Sterol, Acid –base extraction.

دراسة بعض المركبات الكيميائية النباتية الموجودة في نبات لسان الثور البومي الذي ينمو طبيعياً في العراق

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الخلاصة

لسان الثور هو عشب فصلي او معمر، اوراقها ذوات شعيرات، ازهارها منتظمة الشكل. الاسم الشائع لها في العراق (لسان الثور) وهي من عائلة الحمحميات. يحتوي النبات على الفلافونويدات، الاحماض الفينولية، الالكالويدات، الستيرويدات والتيربينويدات. تم ازالة الدهون من اجزاء النبات الكاملة بواسطة تنقيتها لمدة 24 ساعة في الهكسان. تمت عملية الاستخلاص للاجزاء المنزوعة الدهون في محلول الميثانول لحد النفاذ باستخدام جهاز السكسوليت ثم تمت تجزئة المستخلص بعدة مذيبات عضوية ذات قطبية مختلفة شملت بالتتابع: البتروليوم ايثر - كلوروفورم - خلات الاثيل و البيوتانول الاولي. تم كسر الاصرة الكلايكوسيدية باستخدام حامض الهيدروكلوريك 5% لمدة 5 ساعات. تم فصل حامض الروزمارينيك، حامض الكافيين، جنيسيتين وسيليبين من جزء خلات الاثيل بتقنية طبقة السليكا التحضيرية وشخصت بواسطة مطياف الكشف تحت الحمراء، الفصل الملون عالي الكفاءة، اختيار درجة الانصهار وتقنية الفصل الملون بطبقة السليكا الرقيقة. نظرا لاحتواء النبات على الالكالويدات تم استخلاص بواسطة الحامض والقاعدة للحصول على الالكالويدات من مستخلص الخام الناتج من تنقيع النبات في الميثانول (الطريقة الباردة) للحصول على الالكالويد الذي تم فصله بواسطة تقنية طبقة السليكا التحضيرية وشخصت بواسطة تقنية الفصل الملون بطبقة السليكا الرقيقة، مطياف الكشف تحت الحمراء. يهدف البحث الحالي لاجراء دراسة كيمو نباتية للنبات لعدم وجود دراسة سابقة لهذا النوع في العراق.

الكلمات المفتاحية: لسان الثور، حامض الروزمارينيك، الستيرويدات، الاستخلاص بواسطة الحامض والقاعدة.

Introduction

Hardy annual biennial or perennial herb⁽¹⁾, usually with hairs on the leaves, the flowers generally regular⁽²⁾. Commonly named (Lisan Althour) in Iraq, from Boraginaceae family⁽³⁾ that distributed in the temperate, usually in Mediterranean and tropical regions; in Iraq is common on the road sided in middle and southern regions⁽¹⁾.

The plants of Boraginaceae family contain naphthaquinones, flavonoids, terpenoids and phenols. However, these plants also have hepatotoxic pyrrolizidine alkaloids.⁽⁴⁾ Plants of the Boraginaceae family are traditionally used in the

treatment of fever, asthma, kidney stones, wound healing, treatment of arthritis, sprains or dislocation of the joints and bone fractures⁽⁵⁾ Flowers of *Anchusa italica* Retz. and *Anchusa strigosa* L. used as tea: tonic to invalids and children; lower pulsation. It is a substitute of *Anchusa officinalis* used as a diaphoretic and diuretic⁽⁶⁾. In Turkey some *Anchusa* species used traditionally as wound healing and diuretic agent and others as demulcent, expectorant, analgesic, sedative⁽⁷⁾ In Jordan and Palestine the root decoction is used as diuretic, for abdominal pain and for treatment of gastric ulcer, while the leaves juice is applied externally for skin disease, arthritis and wounds^(8,9).

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Different species of *Anchusa* extract have many pharmacological activities ; methanolic extract of *Anchusa officinalis* L .has great antioxidant activity as free radical scavenging due to phenolic and flavonoids content,⁽¹⁰⁾While this activity is great for aqueous extract of *Anchusa strigosa* ⁽¹¹⁾ anti-hyperglycemic activity of *A. strigosa* in diabetic rats showed a significant decrease in the fasting blood glucose and an increase in the serum insulin levels after administration that possibly was either by increasing the pancreatic secretion of insulin from existing β -cells or by release from the bound form ⁽¹²⁾ The uses of *Anchusa strigosa* extract has neuroprotective activity against amyloid toxicity ; reducing levels of amyloid secretion from cells and reduced γ -secretase activity ⁽¹³⁾. The volatile oil of *Anchusa strigosa* L. has strong antibacterial activity against both Gram positive and Gram negative bacteria in a high concentration ⁽¹⁴⁾, the antioxidant activity due to phenolic and flavonoids content of *anchusa italica* may be beneficial in ischemic patients ⁽¹⁵⁾ on the other hand *A. strigosa* aqueous and methanol extracts has anti arthritis activity ⁽¹⁶⁾ also finding that the petroleum ether fraction of *Anchusa strigosa* has effective anti-ulcer profile ⁽¹⁷⁾.

Anchusa strigosa contain many secondary metabolites including pyrolizidine alkaloids with high concentration in the leaves followed by the flowers and finally by the roots such as retrorsine, trachelanthamide, supinidine , platynecine and Heliotridine ^(9,18) ,flavonoids and phenolic acid content of this plant including Catechins, Quercetin,rosmarinic acid caffeic acid and others ^(19,20) ,this plant also contains steroids and terpenoids as tormentic acid. ⁽⁵⁾

This study aim is to carry out a phytochemical study of Iraqi *Anchusa strigosa* for isolation of some phenolic acid,flavonoids and alkaloid.

Experimental Section

Plant material

The whole plant of *Anchusa strigosa* L. of family (Boraginaceae) was collected from Khalow Bazian near Kirkuk during April at the flowering stage and the plant was authenticated by Dr. Abdul Hussein Alkhiat, specialist in plant taxonomy in College of Sciences/ University of Erbil.

Extraction

Anchusa strigosa whole plant parts were cleaned, dried at room temperature for 7 days, pulverized by mechanical milled and then weighed. About (500gm) of powdered plant defatted by maceration in pure n-hexane for 24 hours, filtered through a wattman paper, then filled in the thimble and extracted with sufficient amount of absolute methanol by a Soxhlet extractor until complete exhaustion. This extract was concentrated using rotary evaporator. After complete evaporation of the solvent, dry extract was weighted and dissolved in 350 ml water, partitioned with 350 ml (3times)

petroleum ether, chloroform, ethylacetate and butanol. Each fraction evaporated by rotary evaporator, dry, weighted and revealed for preliminary test. The n-butanol fraction was hydrolyzed by reflex with 10% HCl, and then the hydrolyzed fraction was taken with ethyl acetate then dried for further investigation.

Preliminary phytochemical examination of crude methanolic extract:

To identify the phytochemicals in the methanolic extract, general phytochemical screening was performed ⁽²¹⁾

Alkaloids test by Wagner's reagent

To 2-3 ml filtrate few drops of Wagner's reagent were added to test for the presence of alkaloids; if positive it gives brown yellow precipitate.

Test for polyphenol (ferric chloride test)

Three ml of methanolic extract was mixed with (5 ml) of distilled water. To this solution 4-5 drops of 5% ferric chloride solution was added. Development of bluish black color indicates the presence of polyphenol.

Test for sterols

H₂SO₄ test: one ml of methanolic extract was mixed with (1 ml) acetic acid, to which equal volume of concentrated sulfuric acid was added from the side. The development of blue, green ring at the interface indicates the presence of sterol.

Test for saponins

About 2 gm of the powdered extract was boiled in 20 ml of distilled water in a water bath and filtered, 10 ml of the filtrate mixed with 5 ml of distilled water in test tube and shaken vigorously. The presence of saponins indicated by a characteristic persistent froth at least 1cm in height.

Detection of beta-sitosterole and tormentic acid by thin layer chromatography

Few milligrams from the petroleum ether fraction were suspended in about one ml of absolute methanol, applied to a readymade analytical TLC plate precoated with silica gel GF₂₅₄, and developed in two mobile phases: chloroform: methanol (100:10) and toluene: ethylacetate: chloroform (5:1:4) After development, the plates were allowed to dry at room temperature and the separated spots were detected by Liebermann-Burchard reagent used for identification of steroidal compounds ⁽²²⁾.

Isolation of flavonoids and phenolic acid Compounds by Preparative layer chromatography PLC from the ethyl acetate fraction

Flavonoids and phenolic compounds were isolated by preparative layer chromatography PLC from the ethyl acetate fraction of *A. Strigosa* Preparative silica gel GF₂₅₄ plate of 20×20 cm dimension with a layer thickness of 1cm. reactivated

by heating at 120°C for 15–20 min, then cooled for the application of the sample.

One mobile phase: chloroform: methanol: formic acid (75:20:5) for ethyl acetate fraction was used, placed in jar, the jar was lined with a filter paper closed tightly, and left for saturation.

Sample application was done by dissolving 1g of the sample in absolute methanol and applied to the baseline of preparative PLC plate using capillary tubes, the marked bands were scrapped out of the preparative plate on separated papers using a fine spatula. Each band's powder was introduced in an individual clean and dry conical flask, a sufficient quantity of absolute methanol was added, and the flasks were shaken on a warm water bath, filtered through microporous filter (seaterd) then with filter paper. The solvent was evaporated under reduced pressure using rotary evaporator.

The isolated flavonoid and phenolic compounds from ethyl acetate fraction were identified by HPLC, TLC, FTIR and melting point.

Detection of isolated compounds by thin layer chromatography (TLC)

Analytical TLC was performed for each separated compound by using: chloroform:

methanol: acetic acid (87.5:10:2.5) solvent system for isolated caffeic acid &: chloroform: methanol: formic acid (75:20:5) solvent system for isolated rosmarinic acid to measure the R_f value (retardation factor) for that compound and comparing it with standard material.

Detection of isolated compounds by HPLC analysis

The samples and standard that detected by HPLC include: ethyl acetate, n-butanol fraction before hydrolysis, four isolated bands, rosmarinic acid std., caffeic acid std., apigenine std., kaempferol std., genistein std., silybin std.

Each sample was dissolved in 200 microliter methanol prior to inject in HPLC system.

The separation was achieved on C18 column (Knauer, Germany) (250 -4.6 mm i.d., 5 µm particle size, 80 Å pore size) .

The mobile phase contains 1% aq. acetic acid solution (Solvent A) and acetonitrile (Solvent B), the flow rate was adjusted to 1 ml/min, the column was thermostatically controlled at 280C and the injection volume was kept at 20 µl .

A gradient elution was performed by varying the proportion of solvent B to solvent A as shown below.

Time (min)	Mobile A (%)	Mobile B (%)	Flowrate ml/min
0	90	10	1 ml/min
28	60	40	1 ml/min
39	40	60	1 ml/min
60	10	90	1 ml/min

HPLC chromatograms were detected using a photo diode array UV detector at three different wavelengths (272, 280 and 310 nm).⁽²³⁾

Detection of isolated compounds by FTIR analysis

The isolated compounds subjected to FTIR analysis to identify the functional groups. The following condition and apparatus were used SHIMADZU 3800 FT-IR/ Japan in the Pharmaceutical Chemistry Department at AL Mustansiriyah University / College of Science.

Detection of isolated compounds by melting point

Using melting point apparatus Stuart melting point /SMP30.

Acid-base extraction of alkaloids from crude extract

Part of the crude extract from hot method &all extract obtained from cold method (maceration of whole plant part in absolute methanol for 24 hours) were first defatted with n-hexane and partitioned with water to remove pigment and fatty materials(150 ml *3) then the aqueous part is treated with NH₄OH to pH10 to liberate free alkaloid then equal volume of chloroform is added to separator

funnel partitioned and the lower organic layer was collected & dried then acidified with 5% H₂SO₄ to pH 2., to this layer add NH₄OH to pH10 and partitioned with chloroform, the chloroform layer now contains free tertiary alkaloids⁽²⁴⁾ . This fraction symbolized as FK

Isolation and purification of alkaloid from FK fraction by preparative layer chromatography

The alkaloid isolated by preparative layer chromatography PLC from FK fraction of *A. Strigosa* Preparative silica gel GF₂₅₄ plate of 20 ×20 cm dimension with a layer thickness of 1cm. reactivated by heating at 120oc for 15–20 min, then cooled for the application of the sample. mobile phase: methanol: water: formic acid (25:2:73) was used, placed in jar, the jar was lined with a filter paper closed tightly, and left for saturation. Sample application was done by dissolving 1g of the sample in absolute methanol and applied to the baseline of preparative PLC plate using capillary tubes, the marked bands were scrapped out of the preparative plate on separated papers using a fine spatula. Each band's powder was introduced in an individual clean and dry conical flask, a sufficient quantity of absolute methanol was added, and the flasks were shaken on a warm water bath, filtered through

microporous filter (seaterd) then with filter paper. The solvent was evaporated under reduced pressure using rotary evaporator. The isolated alkaloid then identified by TLC, FTIR.

Detection of isolated compounds by thin layer chromatography (TLC)

Analytical TLC was performed for separated compound by using methanol: water: formic acid (25:2:73) and methanol: water: formic acid (50:50:2) as solvent system for isolated compound then sprayed with Dragendorff reagent for identification of alkaloid.

Detection of isolated compounds by FTIR Analysis

The isolated compounds subjected to FTIR analysis to identify the functional groups. The following condition and apparatus were used SHIMADZU 3800 FT-IR/ Japan in the pharmaceutical chemistry department at Mustansiriya University / college of science.

Results

Phytochemical investigation for methanolic extract of Anchusa strigosa

Table 1 showed the major active constituents present in the crude methanolic extract of *A. strigosa*.

The present study for the *Anchusa strigosa* showed the presence of medicinally active qualitatively analyzed and the results are presented in Table 1. the positive results, based on the presence or absence of

color change, polyphenols, alkaloid, sterols and saponins gave positive (+) results.

Table1. Phytochemical analysis of Anchusa strigosa Extract

Phytochemical components	Result
Alkaloid	+
Polyphenol	+
Sterol	+
Saponin	+

Detection of beta-sitosterole and tormentic acid by thin layer chromatography:

Depending on the color of standards spots that appear after heating and spraying TLC plate with Liebermann- Burchard reagent; beta sitosterol appeared as pink to red color while tormentic acid as purple color.

Isolation of flavonoids and phenolic acid Compounds by Preparative layer chromatography PLC from the ethyl acetate fraction:

Preparative PLC was used to isolate and purify flavonoids & phenolic compounds; using chloroform: methanol: formic acid (75:20:5) solvent system for developing the isolation. As shown in Figure (1), four bands (a,b,c,d) representing the four isolated compounds respectively (R,CA, G,S) .

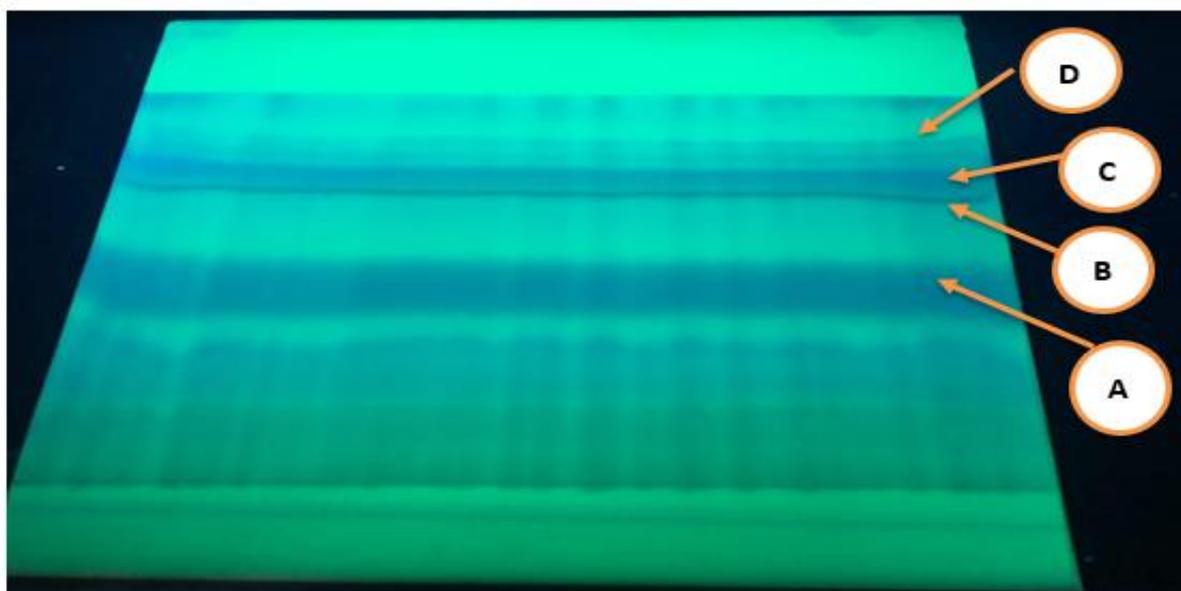


Figure 1. preparative layer chromatography for ethyl acetate fraction developed in chloroform: methanol: formic acid (75:20:5) & detect under UV light at 254 nm (A: R, B:CA, C: G, D: S)

HPLC Chromatogram for the ethyl acetate fraction

HPLC analysis were performed for the ethyl acetate fraction (Figure 2, 3) and each isolated

compound as shown in (Figures 4,5,6,7).

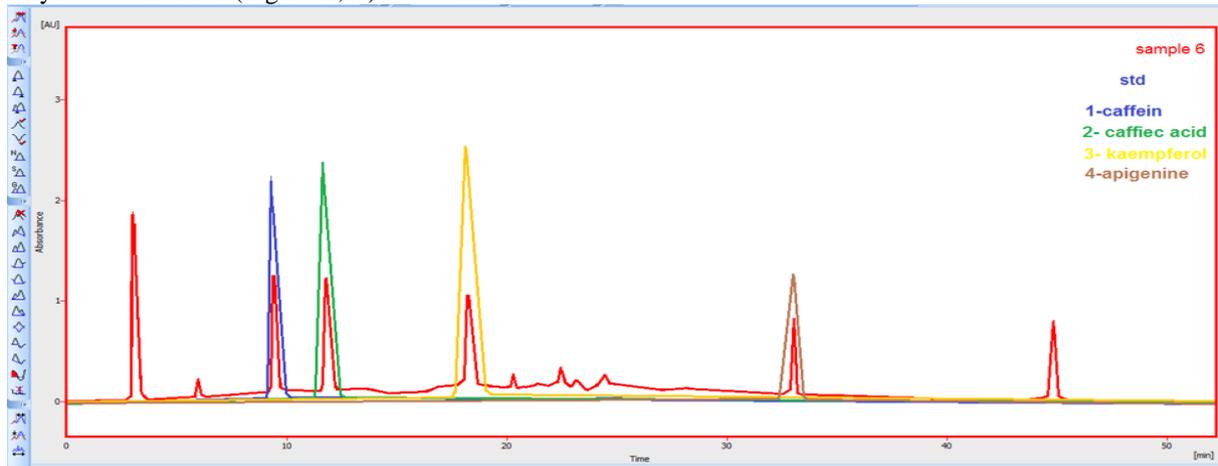


Figure 2. HPLC chromatogram of ethyl acetate fraction.

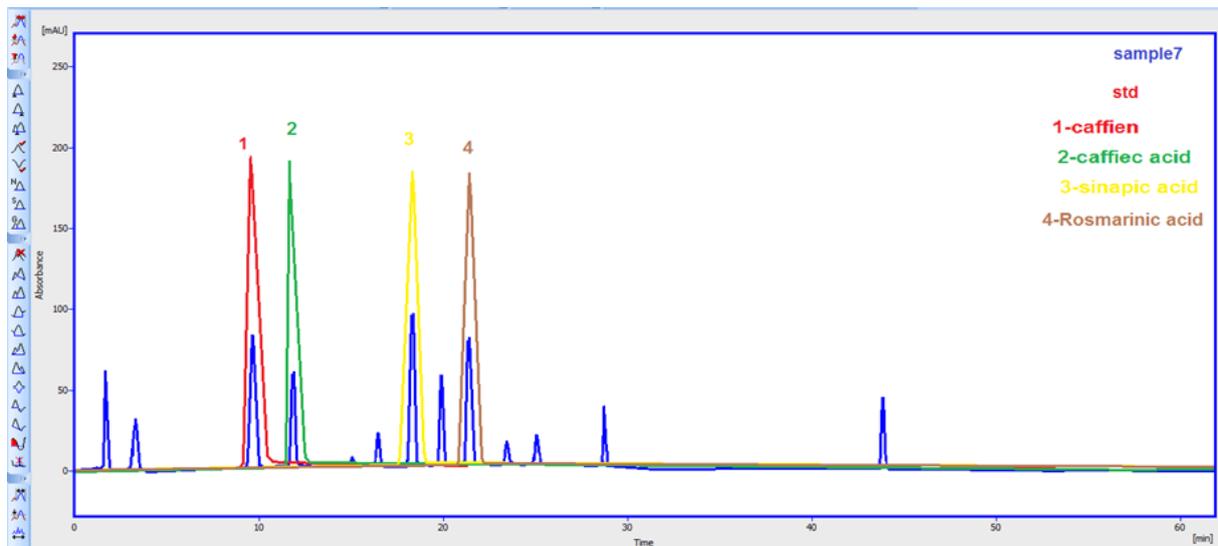


Figure 3. HPLC chromatogram of n- butanol fraction before hydrolysis

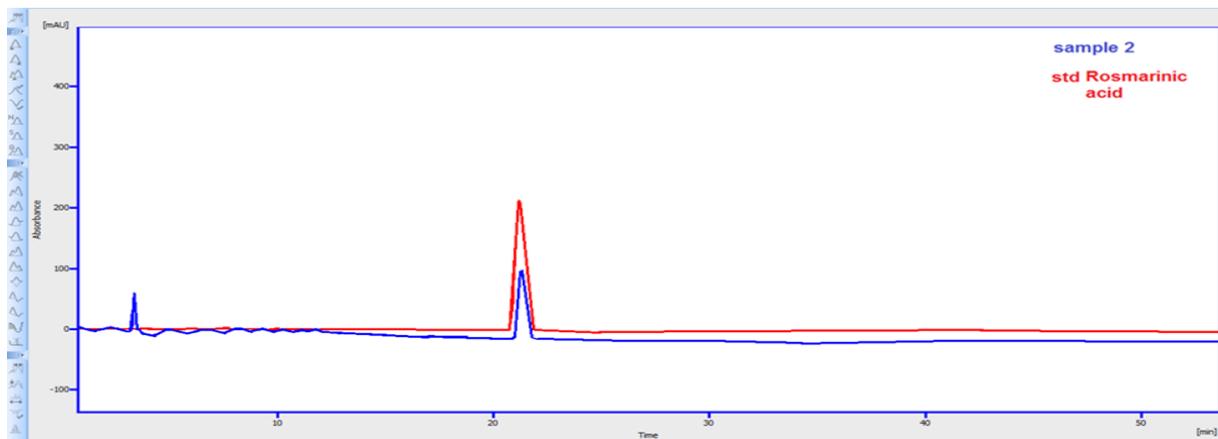


Figure 4. HPLC chromatogram of the isolated R compound & standard rosmarinic acid

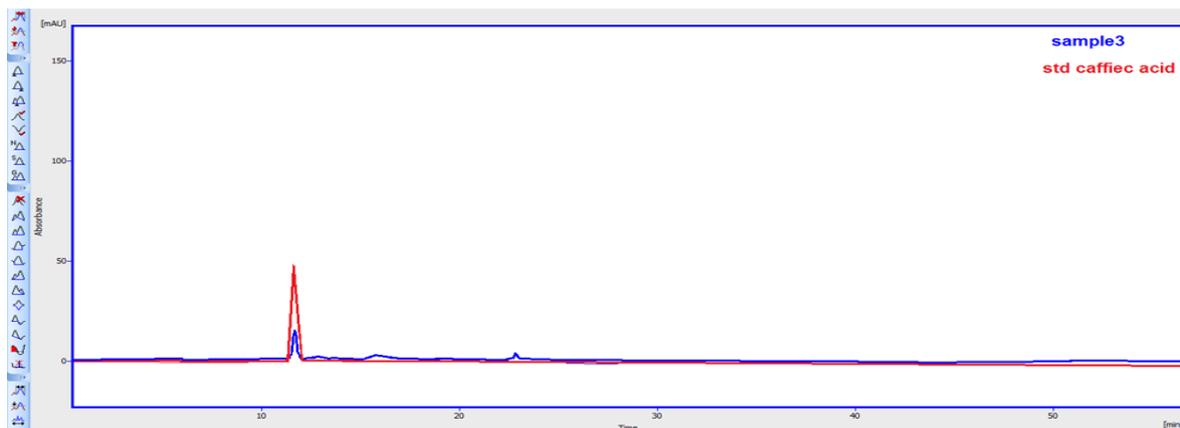


Figure 5. HPLC chromatogram of the isolated CA compound & standard caffeic acid

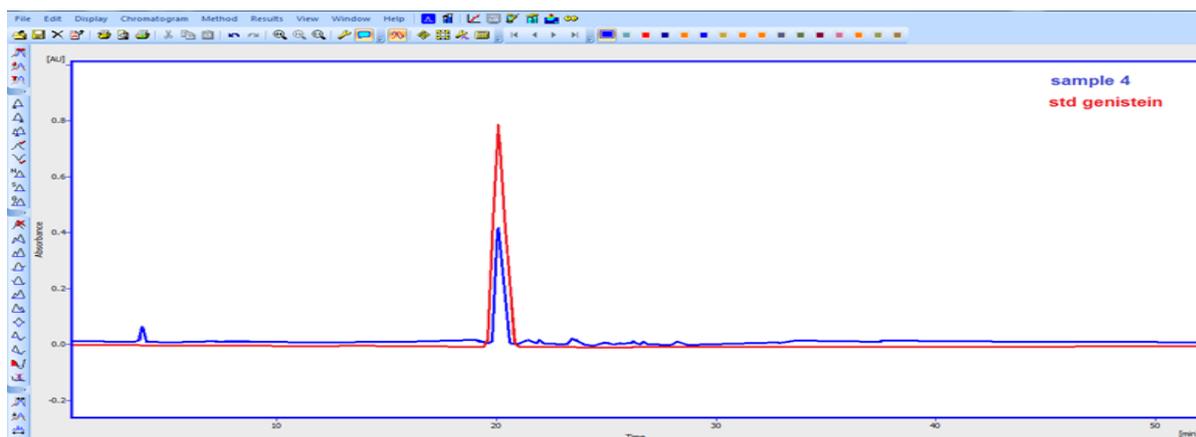


Figure 6. HPLC chromatogram of the isolated G compound & standard genistein

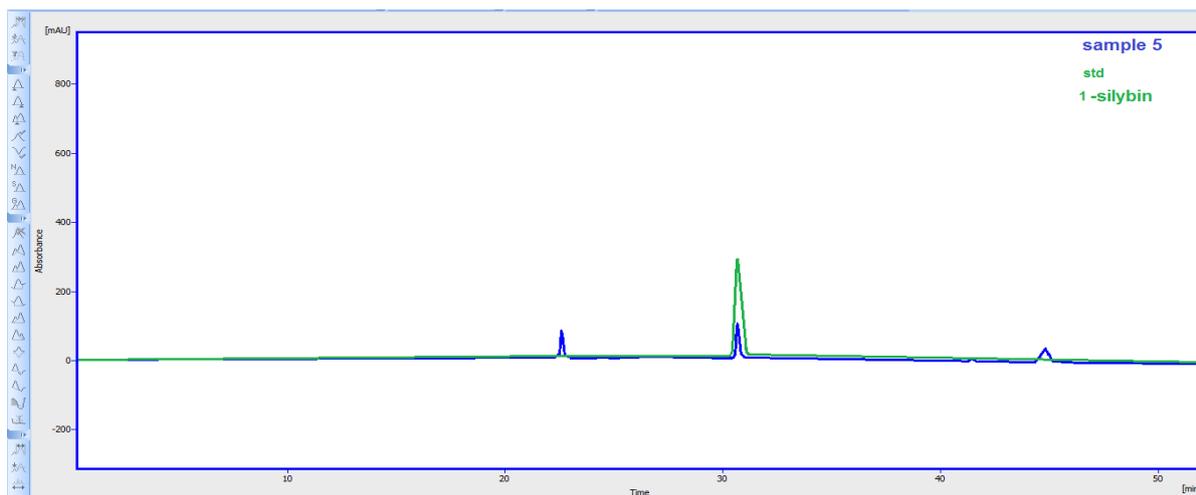


Figure 7. HPLC chromatogram of the isolated S compound & standard silybin

TLC for the isolated compounds

This was carried out to insure the purity of the isolated compound which was isolated by scraping the isolate bands of the preparative PLC by comparing the retardation factor of isolated compound with standard. Retardation factor for isolated R compound compared with that of standard rosmarinic acid after developing the TLC plate in chloroform: methanol: formic acid (75:20:5); they have same R_f value (0.468), retardation factor for isolated CA compound compared with that of standard caffeic acid after developing the TLC plate in chloroform:

methanol:acetic acid (87.5:10:2.5); they have approximate R_f value (0.178 for standard & 0.142 for isolated compound), retardation factor for isolated G compound was (0.837) after developing the TLC plate in chloroform: methanol: formic acid (75:20:5) , retardation factor for isolated S compound; they have approximate R_f value (0.863 for standard & 0.818 for isolated S) after developing the TLC plate in chloroform: methanol: acetic acid (87.5:10:2.5) and all the plates detected at 254 nm UV light.

FTIR Analysis for the isolated compounds

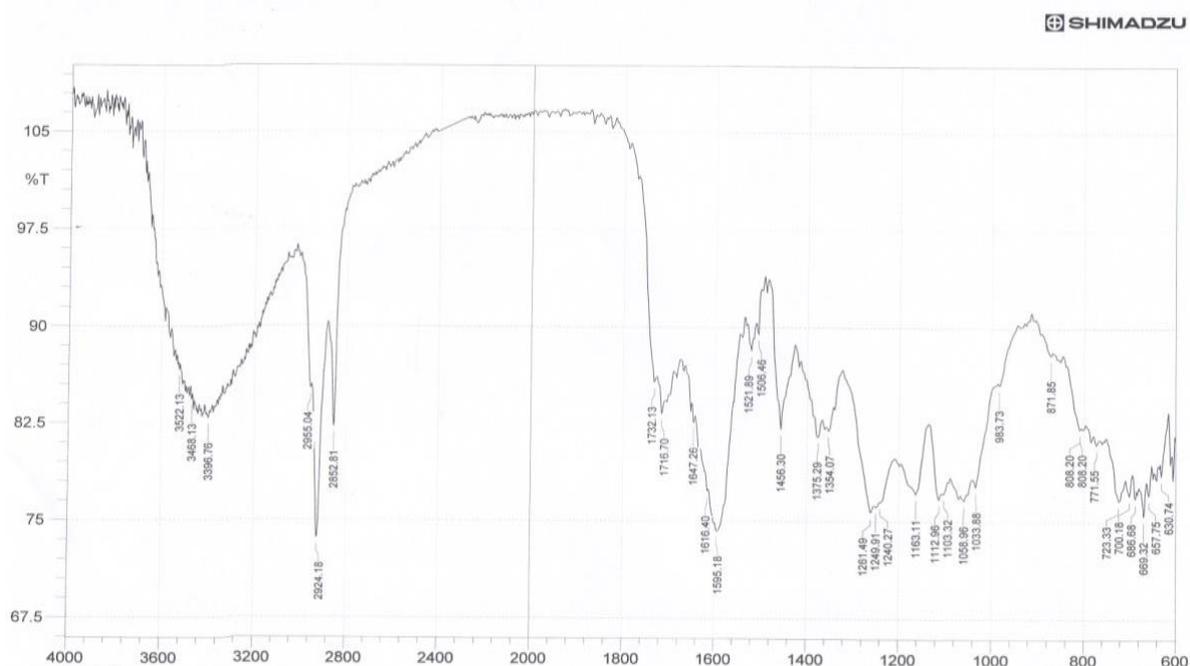


Figure 8. FTIR spectrum of isolated R compound

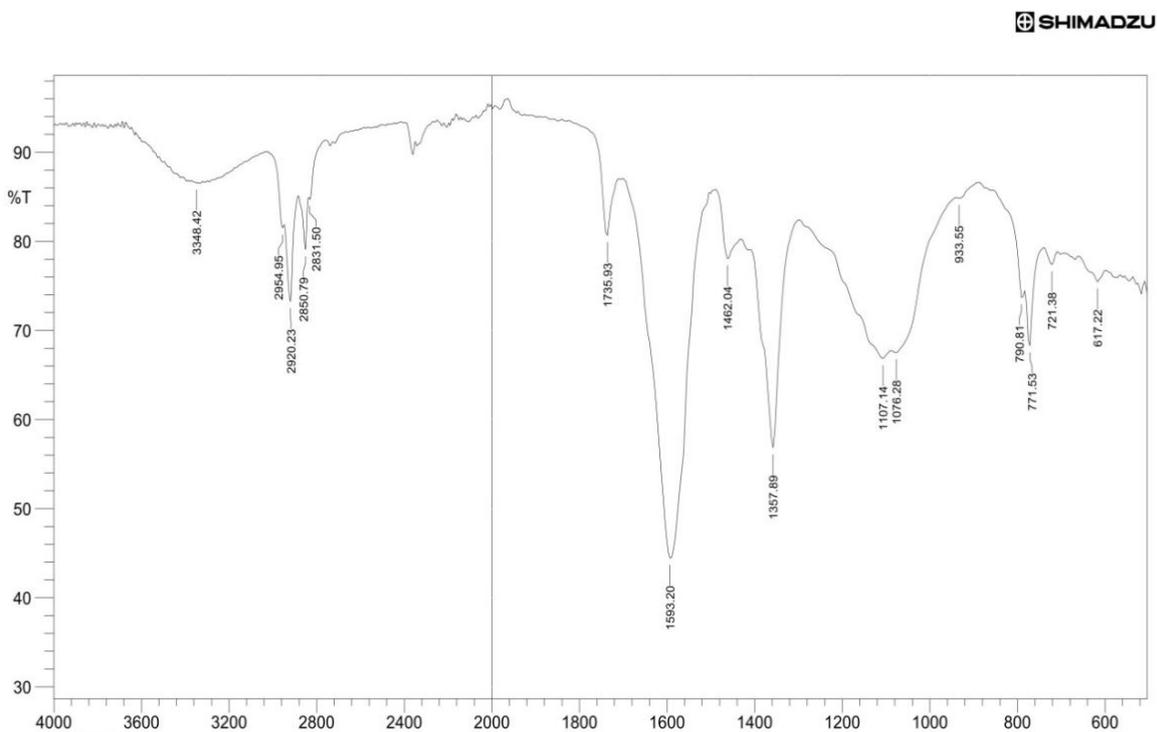


Figure 9. FTIR spectrum of isolated CA compound

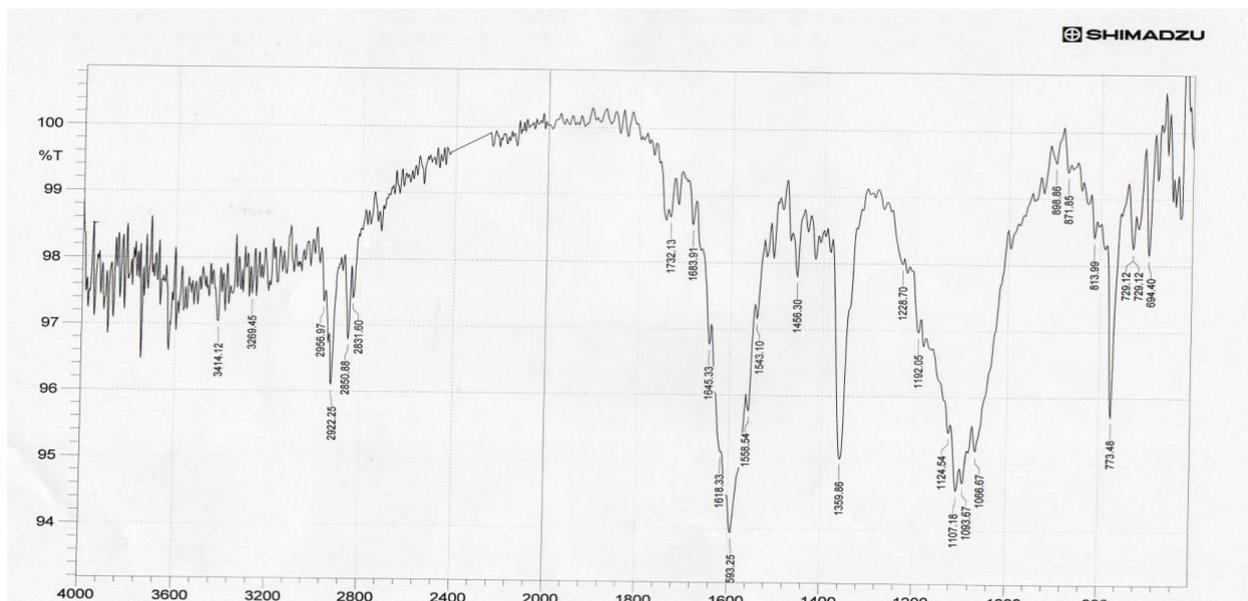


Figure 10. FTIR spectrum of isolated G compound

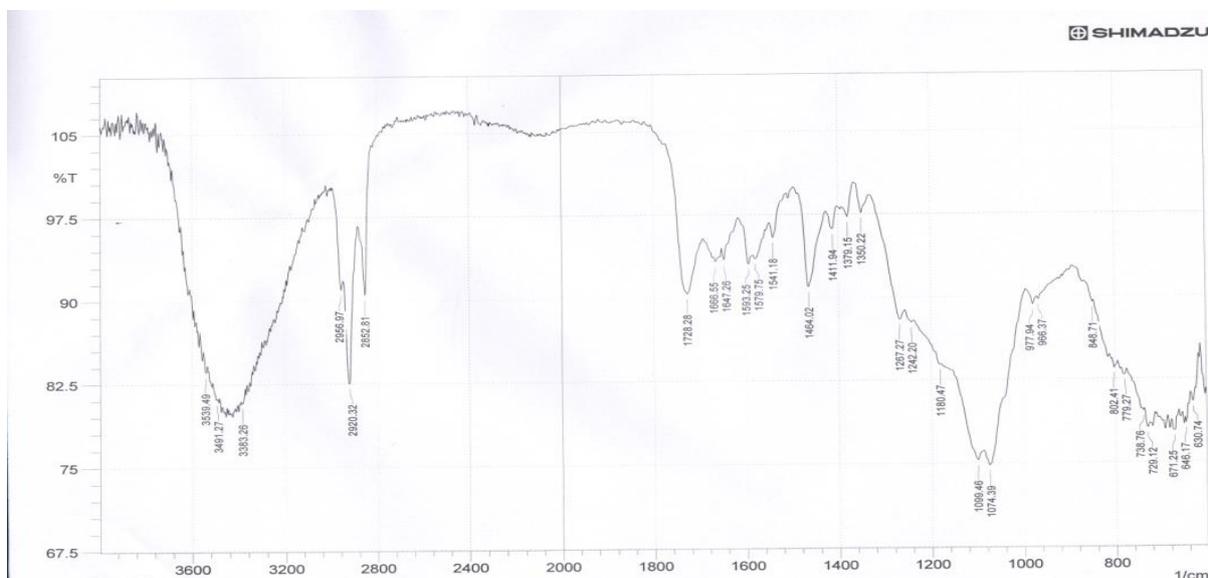


Figure 11. FTIR spectrum of isolated S compound

Melting point

- Isolated R compound melt at 171 to 175 °C which match standard rosmarinic acid.
- Isolated CA compound melt at 221-224 °C which match standard caffeic acid.
- Isolated G compound melt at 298- 300 °C which match standard genistein.
- Isolated S compound melt at 162–163°C which match standard silybin.

Isolation and purification of alkaloid from FK fraction by preparative layer chromatography

Preparative PLC was used to isolate and purify the compound that detected as alkaloid by spraying the developing plate by dragendorff reagent;

methanol: water: formic acid (25:2:73) was used as solvent system for developing the isolation.

Detection of isolated compound by thin layer chromatography (TLC)

This was performed to insure the purity of the isolated compound which identified by spraying the plate with dragendorff reagent and measuring the retardation factor (R_f) after developing the TLC plate ; founding the R_f value was (0.193) by using methanol: water: formic acid(25:2:73) and was (0.654) by using methanol: water: formic acid (50:2:50) as solvent system.

FTIR Analysis for the isolated compound

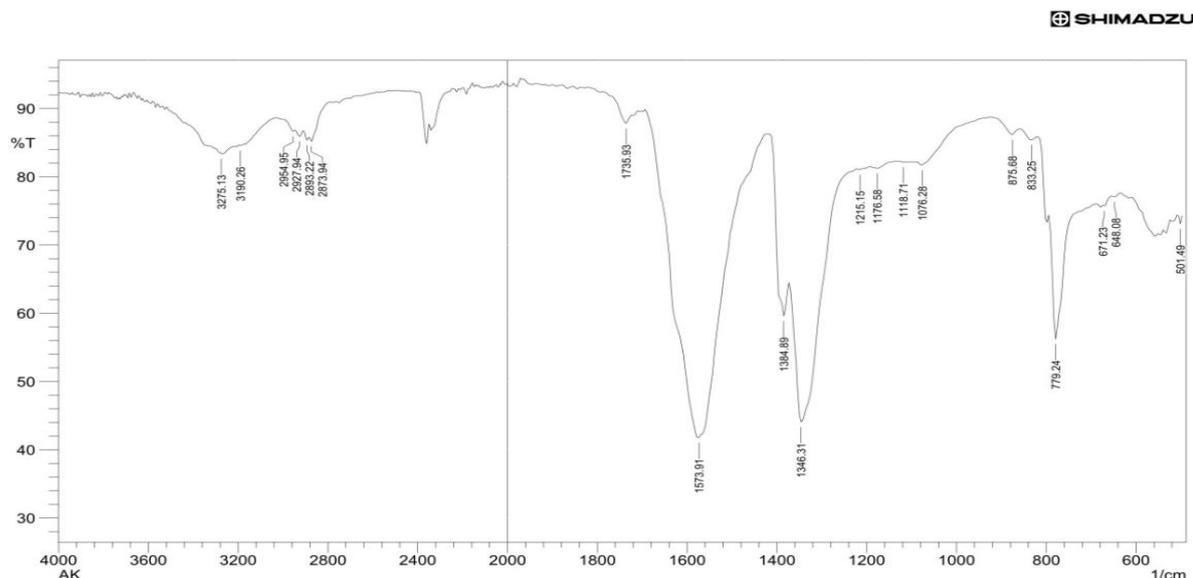


Figure 16. FTIR spectrum of isolated ak compound

Discussion

HPLC, IR analysis and melting point carried out in this study to identify and confirm the presence of rosmarinic acid, caffeic acid, genistein and silybin, and pyrolizidine alkaloid in the Iraqi *Anchusa strigosa*.

Conclusion

The results of this study exhibited the presence of phenols i.e., rosmarinic acid, caffeic acid and flavonoids, i.e., genistein, silybin in ethyl acetate fraction, pyrolizidine alkaloid after acid – base extraction of crude plant material.

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