

Extraction and Identification of Phenolic Compounds from the Iraqi *Heliotropium europaeum* L. plant

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Abstract

The *Heliotropium europaeum* L. (Boraginaceae) are well-known to contain toxic pyrrolizidine alkaloids in addition to other secondary metabolites. Its spread in the Mediterranean area, southern Europe, Central Asia, and Iraq. The present study included extraction of the aerial parts of Iraqi *Heliotropium europaeum* with methanol (soxhlet apparatus), fractionation, screening the active constituent, and identification by chromatographic techniques. The extract was suspended in distilled water and partitioned with chloroform, ethyl acetate, and n-butanol. The hydrolysis step was done for the two fractions (n-butanol and ethyl acetate). Phytochemical screening and identification of bioactive substances of the plant was done for the two fractions. A qualitative and quantitative analysis of the fractions was carried using a high-performance liquid chromatography technique and twenty-one reference standards. The outcomes of this study were the identifications of new six phenolic compounds (kaempferol (1), Silybin (2), caffeic acid (3), Genistein (4), Apigenin (5), in addition to syringic acid (6)) from *Heliotropium europaeum* ethyl acetate fraction.

Key words: *Heliotropium europaeum*, soxhelt, Genistein, syringic acid, High-performance liquid chromatography.

فصل وتشخيص المركبات الفينولية من النبات العراقي حشيشة العقرب

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الخلاصة

نبات حشيشة العقرب أو التنوم من عائلة (Boraginaceae) معروف باحتوائه على قلويدات بيروليزيديين السامة بالإضافة إلى نواتج الأيض الثانوية الأخرى. ينتشر في منطقة البحر الأبيض المتوسط وجنوب أوروبا وآسيا الوسطى والعراق. تتضمن الدراسة الحالية على استخلاص الأجزاء الهوائية من الهليوتروبيوم العراقي بالميثانول (جهاز سوكلت)، وتم إجراء عملية التجزئة، وفحص المكون النشط، والتعرف على التقنيات الكروماتوجرافية. تم تعليق المستخلص في ماء مقطر وتم تقسيمه باستخدام الكلوروفورم، وأسيتات الإيثيل ن-بيوتانول. تم إجراء خطوة التحلل المائي للجزيين (ن-بيوتانول وأسيتات الإيثيل). تم إجراء فحص كيميائي نباتي وتحديد المواد النشطة بيولوجيًا في النبات للجزيين. تم إجراء تحليل نوعي وكمي للأجزاء باستخدام تقنية كروماتوجرافيا سائلة عالية الأداء وواحد وعشرون معيارًا مرجعيًا. كانت نتائج هذه الدراسة هي تحديد ستة مركبات فينولية جديدة (كامفيرول (1)، سليبين (2)، حمض الكافيك (3)، (4) جنستين، (5) أبجنن، بالإضافة إلى حمض الحقن (6)) من جزء أسيتات الإيثيل لنبات حشيشة العقرب.

الكلمات المفتاحية: هليوتروبيوم يوروبيوم، سوكلت، جينستين، حمض الحقن، كروماتوجرافيا السائل عالية الأداء.

Introduction

Large number of plants of *Heliotropium* genus belong to Boraginaceae family which is biggest family of plant that includes around 250-300 species in the world. These plant species are widespread in temperate and tropical parts of both hemispheres⁽¹²⁾ Figure 1. The name "heliotrope" is taken from the fact that these plants move their leaves toward the sun^(3,4). The phytochemical studies about *Heliotropium europaeum* are very limited and majority of these studies are concentrated on pyrrolizidine alkaloids (PAs)^(5,6). The toxicity of this type of alkaloids are concentrated usually in the flowering parts of the plants and in the seeds of the Boraginaceae (all genera)⁽⁷⁾. Some Pyrrolizidine alkaloids-bearing plants are used as ornamental plants, ground cover,

soil improvers, and can contaminate feeds of animal⁽⁸⁾. Risks of exposure to Pyrrolizidine alkaloids, in the large countries, originate from so called herbal remedies, folk medicines and herbal teas⁽⁷⁾. Pyrrolizidine alkaloids (PAs) are produce by plants and regarded as one of the widespread toxins of natural origin^(9, 10). They are esters produced from esterification of various amino alcohol bases called necine and mono or dicarboxylic acids⁽¹¹⁾. Toxicological studies displayed the reason behind such toxicity of *H. europaeum* is the alkylation that take place by some biological nucleophiles such as nucleic acids (enzymes, DNA and RNA) and proteins via 1,2-unsaturated PAs metabolites^(12,13). The importance of this plant lead to screen the chemical constituents especially the phenolic compounds.

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Figure 1. The Iraqi *Heliotropium europaeum*

Materials and Methods

Plant materials

The aerial parts of *Heliotropium europaeum* L. were collected from the bank of Tigris river in the periphery of Tikrit city in August 2019. The collected plant purified carefully then identified, authenticated by Dr. Khansaa Rasheed at Iraq natural history research center and museum plant and environment department / University of Baghdad. The aerial parts were cleaned, dried in shade, and pulverized in a mechanical grinder to a fine powder

Extraction and fractionation of the different active constituents

100 g of powdered plant was first defatted with hexane (250 mL) for 24 hr. at room temperature (about 25 °C) then filtered. The residual plant was dried on air and packed in the thimble of soxhlet apparatus. The extraction was carried with 750 mL of methanol till exhaustion. The extract was filtered and the solvent was evaporated by applying a reduced pressure by a rotary evaporator. A dark greenish residue was obtained and then suspended in 150 mL distilled water and partitioned with three solvent chloroform, ethyl acetate, and n-butanol (3x150 mL) for each solvent. The chloroform and ethyl acetate fraction were dried with sodium sulfate (anhydrous) then filtered and evaporated to dryness.

Hydrolysis of Ethyl acetate and n-Butanol fractions

The hydrolysis step was done for the two fractions by reflux for 10 hr. using 5% HCl (200 mL). The cooled solution was partitioned by extraction with ethyl acetate (3x200 mL). The layers were combined, dried and filtered. The identification of n-butanol and ethyl acetate fractions were carried out by thin layer chromatography (TLC) and HPLC technique.

Identification of phenolic compounds in *Heliotropium europaeum* plant extract

1. Preliminary phytochemical analysis by chemical tests

Preliminary qualitative phytochemical analysis for the screening and identification of some important chemical constituents of *Heliotropium europaeum* plant under study was carried out on crude extract, and fractions using the standard procedures as previous reported⁽¹⁴⁾.

Test for phenolic compound: The plant extract (or fractions) (500 mg) was dissolved in 80% methanol (20mL) and filtered. The filtrate was used for the following tests:

(a) NaOH test: The alkaline reagent test for flavonoids was performed by placing 0.5 mg of the crude alcoholic extract in a test tube followed by addition of diluted solution of 10% sodium hydroxide drop wise. The dissolved flavonoids formed an intense yellow color due to formation of sodium phenolate salt as precipitate. This color was changed to faint yellow upon the addition of diluted solution of hydrochloric acid HCl. It was noted that any further addition of diluted solution of HCl to the resulted solution, turns the solution to colorless that indicate the flavonoids presence^(15,16).

(b) Ferric chloride test: the plant extract (25 mg) dissolves in distilled water (10mL) and filter. Aqueous ferric chloride (1% FeCl₃) solution was added to the filtrate. The appearance of intense green, black or blue color indicates the of phenolic acid presence in the sample due to formation of ferric phenolate salt as precipitate.

2. Thin layer chromatography

Using readymade plate aluminum coated TLC sheet G/F₂₅₄, 0.20 mm stationary phase used was silica gel, using UV light for detection of the spot. Different mobile phases were tried for the detection of plant constituents (phenolic compounds) and it was used for the identifying of phenolic compounds of *Heliotropium europaeum* L that found in ethyl acetate and n-butanol fractions of the plant.

The P1 mobile phase (CHCl₃: acetone: formic acid (75:16.5:8.5)⁽¹⁷⁾ proved to be the best for separation of the fractions, and it was adopted for separation. After drying the developed plates were sprayed with 5% ethanolic KOH.

3. The HPLC technique

The HPLC conditions for analyzed fractions

HPLC system instruments were supplied by Knauer, Germany.

Table 1. HPLC System components.

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

In this method a C18 column was used for separation with internal diameter of (250 - 4.6 mm). The particle size is 5 µm with a pore size of 80 Å⁽¹⁸⁾.

1% aqueous acetic acid, solvent A, and acetonitrile, Solvent B, were the mobile phases. Moreover 1 ml/min was the flow rate. The column was thermostatically controlled at 28°C and the injection volume was fixed at 20 µL.

The technique was employed was gradient elution with continuous change of B solvent proportionally to A solvent. Table (2). The instrument is equipped with photo diode UV detector at the following wave lengths (272, 280 and 310 nm) in order to scanned the resulted chromatogram.

Table 2. The gradient program used in separation by HPLC system.

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

The detection of each metabolite was performed by matching retention time and absorbance spectrum of the standards. For quantitative analysis the concentration components were calculated by calibration curve in which serial concentrations of reference standards was plotted against their equivalent peak area, the calculation was done by using the straight line equation $Y = aX + b$, $slop = y/x$ (Y and X: are holding the place of coordinates (x, y) of any point that lies on the line, a: slop, b: y-intercept), where R²: regression factor. Twenty-one standards were used in this study and they were:

Quercetin, Apigenin, Luteolin, Chlorogenic acid, Kaempferol, Caffeic acid, Genistein, Ferullic acid, Cinnamic acid, Silybin, Oleuropein, Caffeine, Myricetin, Salicylic acid, Sinapic acids, Curcumin, Demethoxycurcumin, Bisdemethoxycurcumin, Daidzein, Syringic acid, Gallic acid.

Preparation of stock solutions for standards compounds and the examined samples for HPLC

Stock solutions used in HPLC analysis were prepared from 0.04 mg of each standard in 1mL methanol (HPLC grade). The operator data were obtained from the database of the instrument. The examine samples were subjected to ultrasonication (Baransonsonifier, USA) at 60% duty cycle at 25°C for 30 minutes. The resulted suspension was centrifuge (at 7500rpm) 20 min. The clear upper layer of sample solution was separated then evaporated under vacuum. The residue was dissolved in 1mL methanol using vortex mixer followed by filtration through disposable filter (2.5 µm). The clear solution temperature adjusted to 4°C. The used volume for injection of the sample was 20 µL.

Results and Discussion

In spite of the importance of *H. europaeum* as a toxic plant, the phytochemical studies concentrated on the alkaloids secondary metabolites especially pyrrolizidine type. Therefore, the chosen of phenolic compounds in this study is the main target because they have different pharmacological activities.

In this study, hot extraction method was done by absolute methanol to extract the active constituent depend on the nature of these active constituents.

The hydrolysis process was done to remove the glycoside moiety from the compounds by cleavage the ether linkage (glycosidic bond) producing the free a glycon. The screening of these sample revealed that they contain various phytochemical constituents by using p1 solvent system (Chloroform: acetone formic acid) (75:16.5:8.5) which is best mixture of solvents used in the separation of phenolic compound from fraction and 254 nm and 366 nm UV light.

Identification of phenolic compounds in Heliotropium europaeum plant extract

1- Preliminary phytochemical investigation like chemical tests were carried on the *H. europaeum* aerial parts and showed the following results as shown in Table 3 .

Table 3. Qualitative analysis of phytochemical constituents in crude extract of Heliotropium europaeum L.

Name of test	NaOH test	Ferric chloride test
Fraction		
Ethyl acetate	+ve	+ve
n-butanol	+ve	+ve

2- Preparative Thin Layer Chromatography analysis for the ethyl acetate and n- butanol extract.

Preparative Thin Layer Chromatography technique was used for separation of small amount constituent by using the same separation condition that was followed previously in ordinary TLC technique. The results of preparative TLC were

more than one bands in each fraction which represent that the sample contain many of chemical constituents which can be separated easily. Figure (2-3).

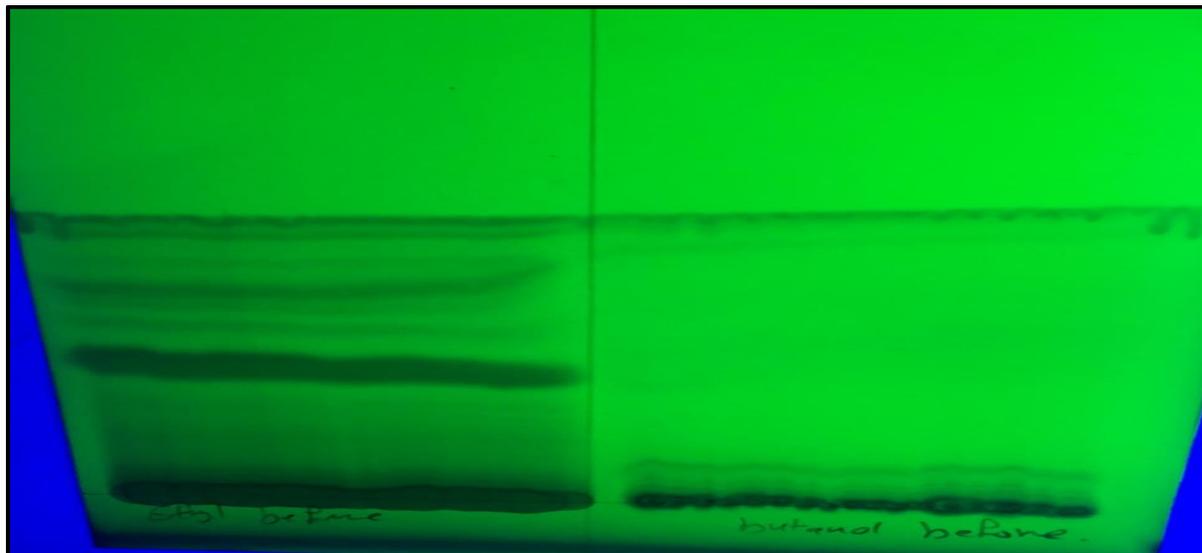


Figure 2. Preparative Thin layer chromatography on silica gel GF₂₅₄ for fractions

(1) Fraction of ethyl acetate before hydrolysis (2) fraction of n-butanol before hydrolysis. Using solvent system P1 (CHCl₃:CH₃OCH₃: HCOOH) in a ratio (75:16.5:8.5). The detection under UV light 365 and 254 nm, P1 which is best mixture of solvents used in the separation of phenolic compound from fractions.

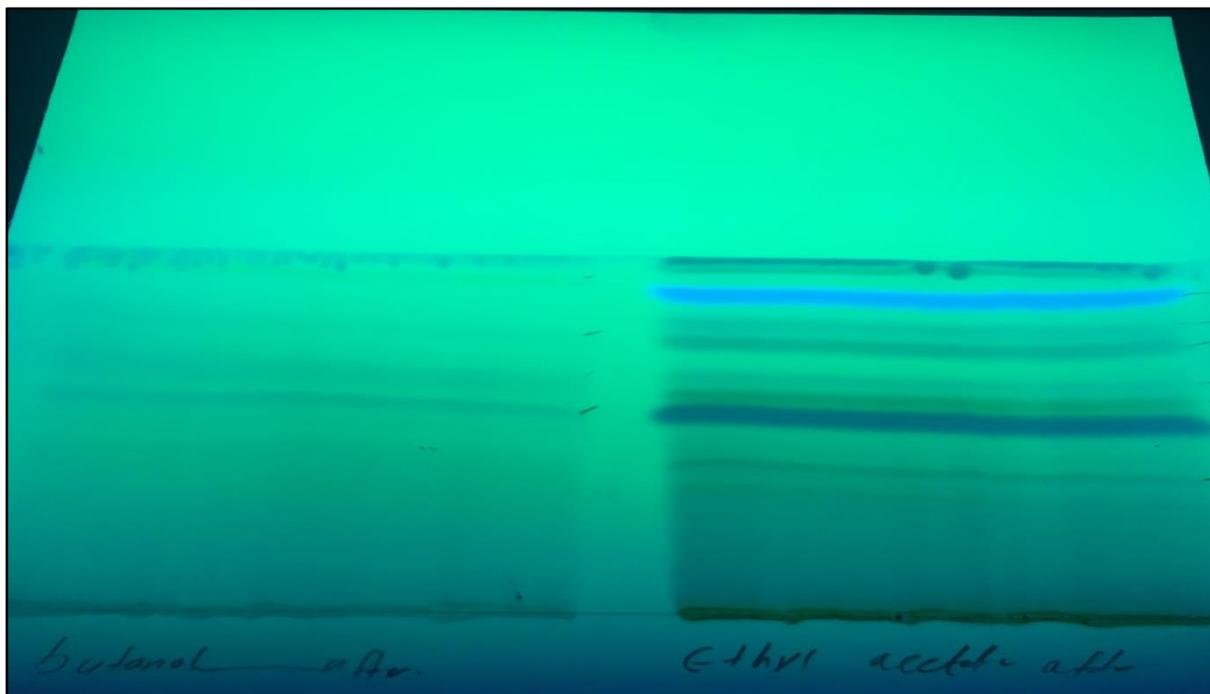


Figure 3. preparative Thin layer chromatography on silica gel GF₂₅₄ for fractions

(1) Fraction of ethyl acetate after hydrolysis (2) fraction of n-butanol after hydrolysis. Using solvent system P1(CHCl₃:CH₃OCH₃: HCOOH) in a ratio (75:16.5:8.5). The detection under UV light 365 and 254 nm.

High-performance liquid chromatography (HPLC)

HPLC technique can provide a lot of information about the content of the extract. It is also used for qualitative identification of extract

constituents by making a comparison of their retention time and the shape of the UV spectrum of the detected compound by the detector of the instrument with that for authenticated reference standards at identical chromatographic conditions.

In this study, the sample was analyzed by this technique, ethyl acetate fraction after hydrolysis (ETA). The resulted chromatogram from fraction was compared with twenty-one standards at the

same condition. Figure (4) shows the HPLC chromatogram of ethyl acetate fraction (ETA) matched with six of the used standards.

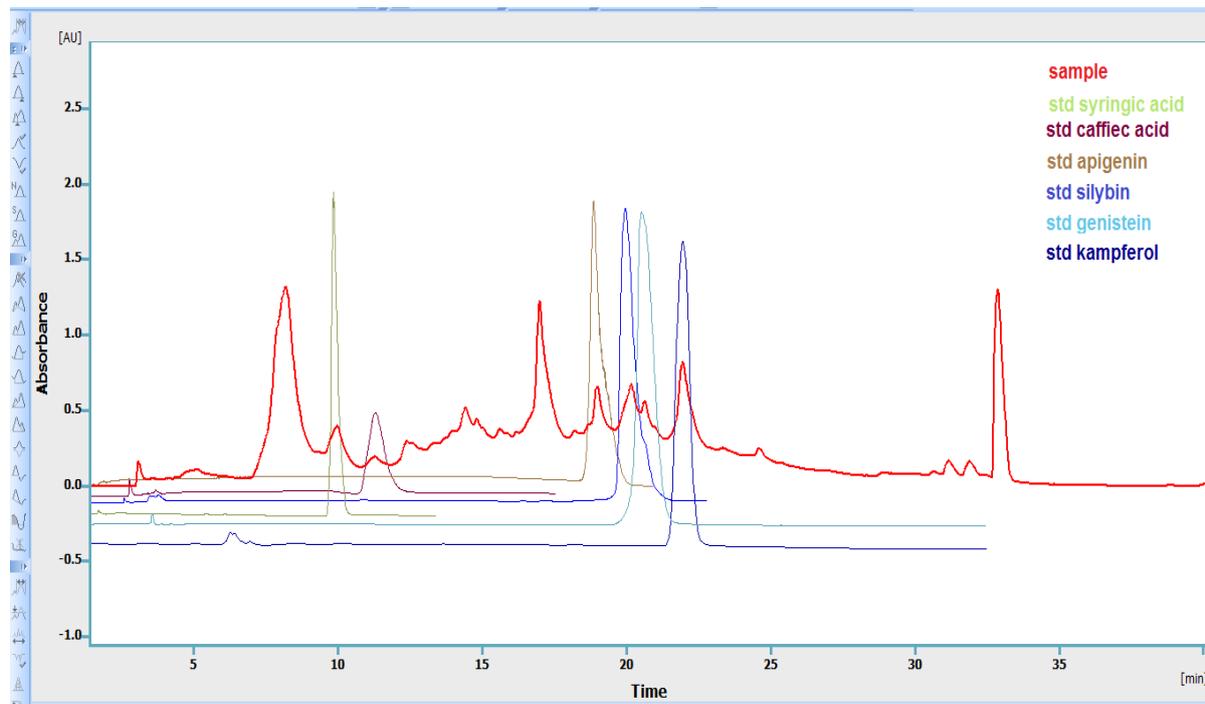


Figure 4. HPLC chromatogram for ethyl acetate fraction (ETA) matched with six detected standards chromatograms.

The matching between the spectra of the detected compounds in ethyl acetate fraction with corresponding standards spectra as shown in Figures (5-10) respectively.

results concluded that *Heliotropium* plant ethyl acetate might contains syringic acid, silybin, kaempferol, apigenin, caffeic acid and genistein.

These figures show an excellent fitness between the detected compounds and the six standards. These

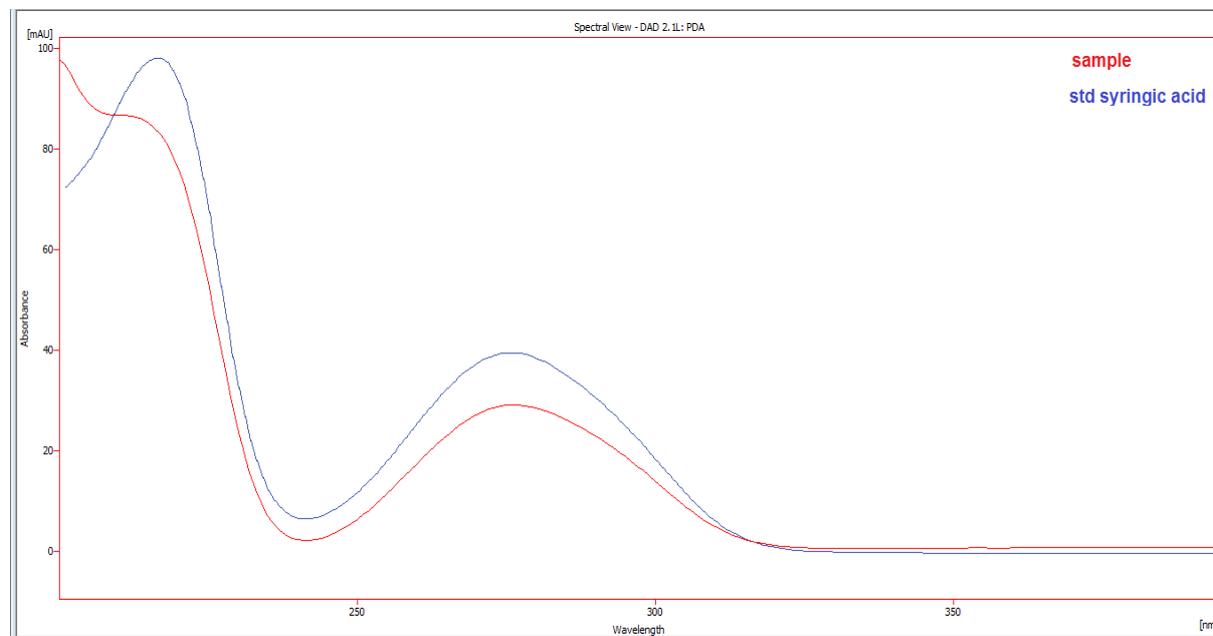


Figure 5. UV spectrum of fraction of ethyl acetate matched with syringic acid standard UV spectrum.

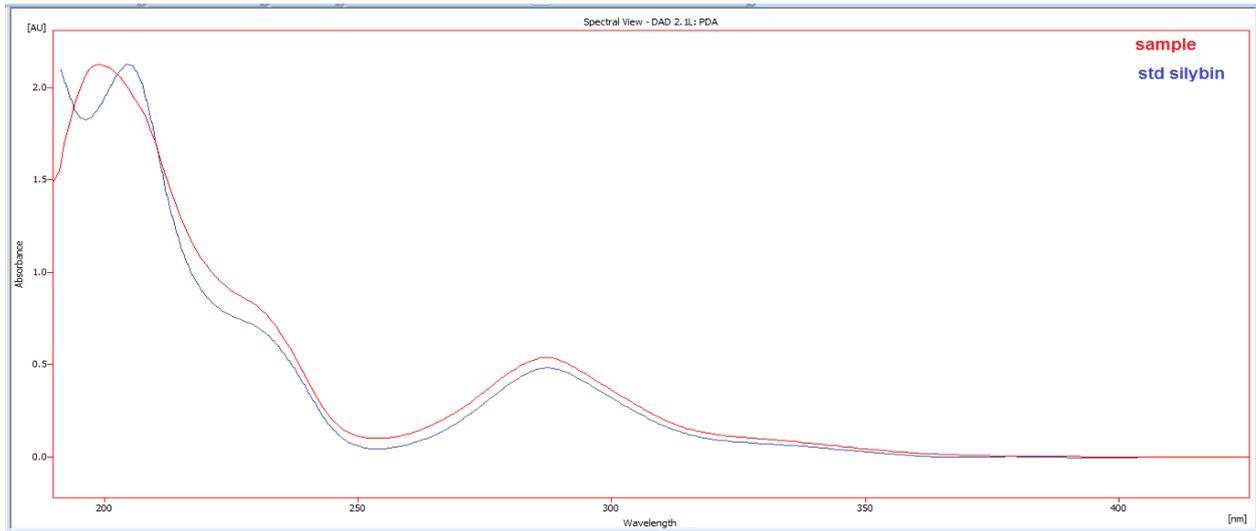


Figure 6. UV spectrum of fraction of ethyl acetate matched with silybin standard UV spectrum.

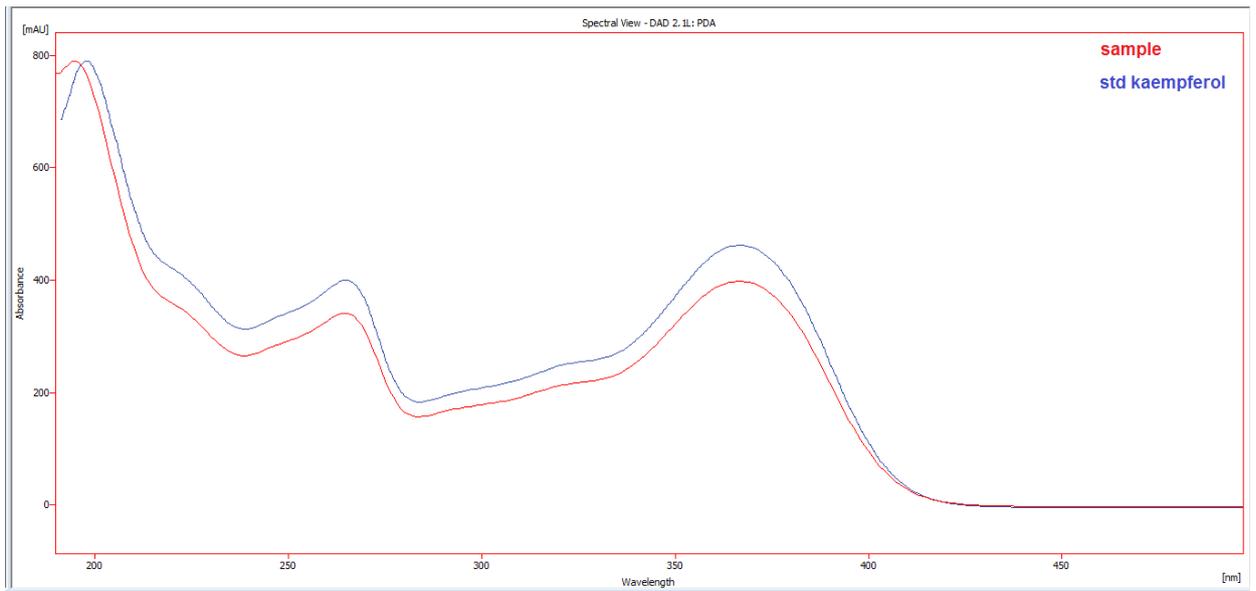


Figure 7. UV spectrum of fraction of ethyl acetate matched with kaempferol standard UV spectrum.

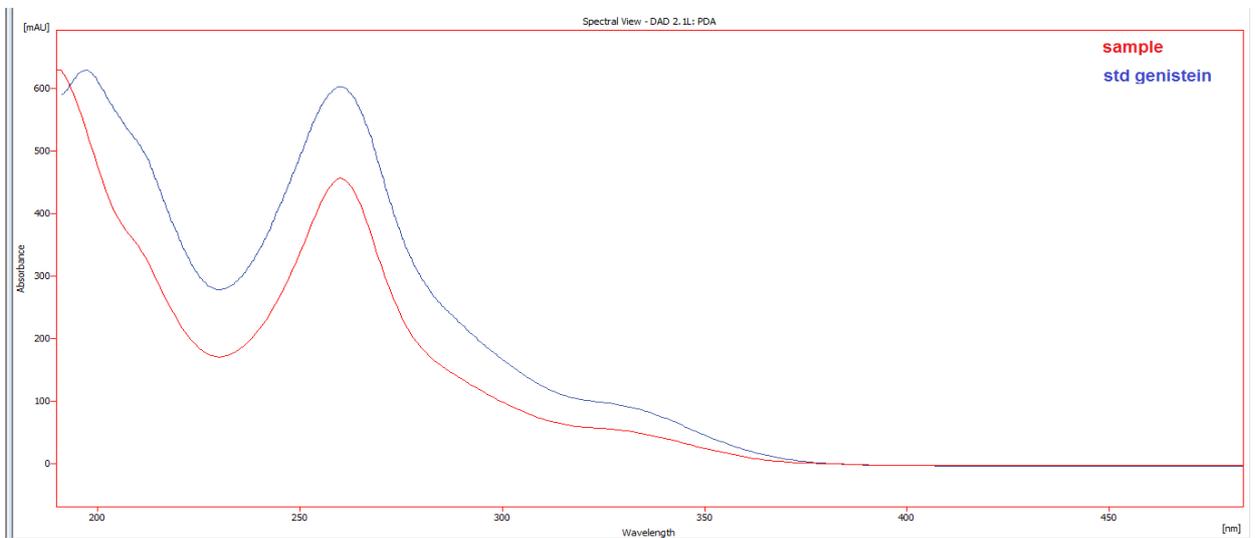


Figure 8. UV spectrum of fraction of ethyl acetate matched with genistein standard UV spectrum.

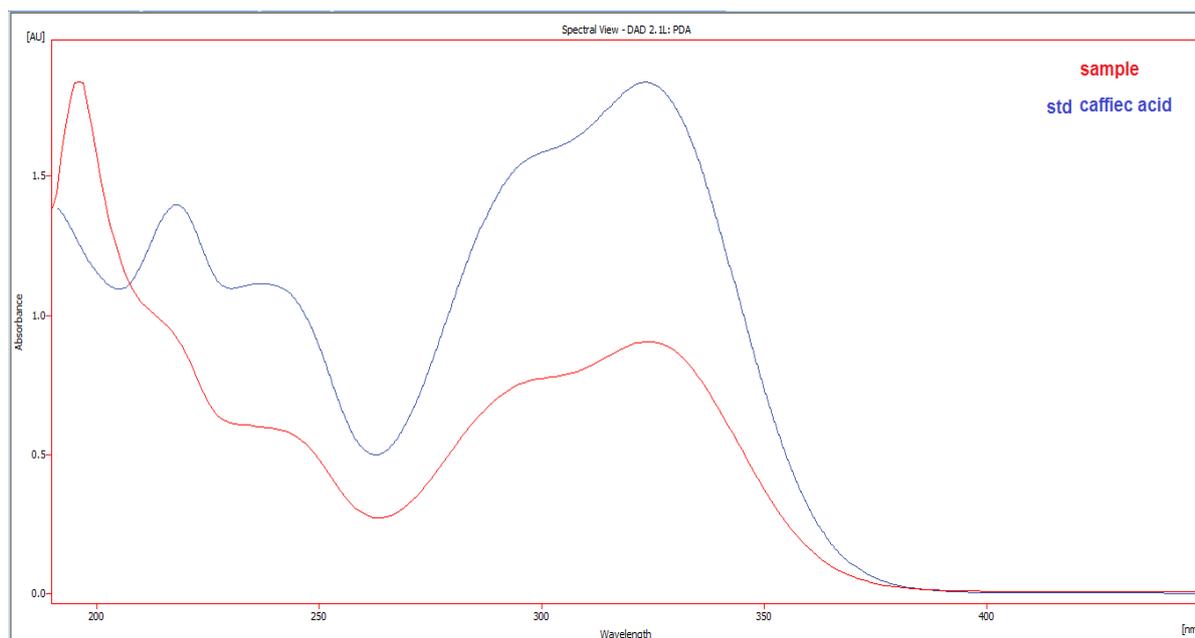


Figure 9. UV spectrum of ethyl acetate fraction matched with caffeic acid standard UV spectrum.

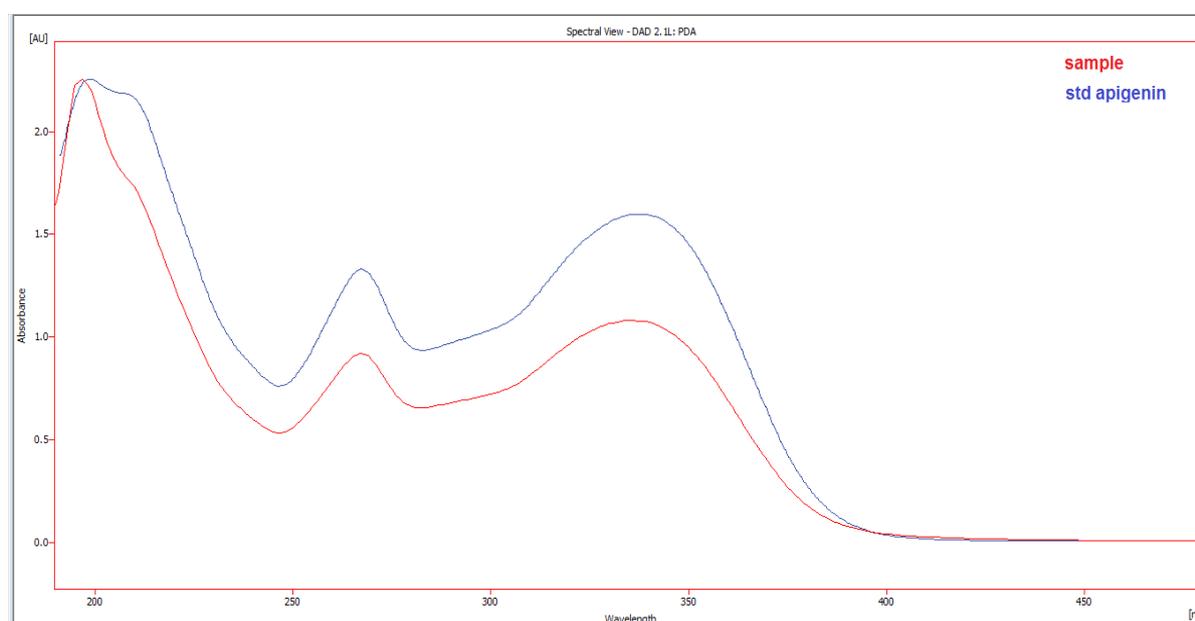


Figure 10. UV spectrum of fraction of ethyl acetate matched with apigenin standard UV spectrum.

It is important to refer that the ethyl acetate fraction chromatogram has at least three significant compounds did not match with the used standards. This reflects the need to extend study by using additional standard. Because in HPLC technique, the area under the peak of a certain compound is proportional to its concentration therefore calibration curves of the six matched standards were constructed for quantitative analysis and the peak areas of the detected compounds in fraction of ethyl

acetate were used to determine the concentration of each one in the fraction, according to the first line equation $Y = aX + b$, $slop = Y$ (area under the curve)/ X (concentration mg/mL).

The quantitative concentration of the six phenolic compounds in ethyl acetate fraction revealed that kaempferol has the highest concentration while genistein concentration was the least as shown in Table 4.

Table 4. The HPLC Quantitative analysis for Ethyl acetate fraction.

Ethyl acetate fraction	peak area (y)	µg/ml (x)	mg/ml
Kaempferol	5327.835	111.39857	0.110399
Silybin	3695.17	64.995163	0.064995
caffeic acid	1977.472	65.9707089	0.065971
Genistein	1209.131	6.22993652	0.00123
Apigenin	446.711	19.8538222	0.019854
syringic acid	7812.712	89.8808371	0.089881

From the above finding, *Heliotropium europaeum* is a promising plant as it contains different secondary metabolites especially phenolic compounds that was detected novelty in this study

Conclusion

Little attention was reported about the toxic *H. europaeum* plant and its secondary metabolites especially its phenolic compounds. Ethyl acetate and n- butanol fractions of this plant were prepared and their phenolic compounds were studied. The technique used was high performance liquid chromatography equipped with UV-vis facility and group of standard phenolic compounds. Six phenolic compounds were isolated and identified for the first time from ethyl acetate extract of this plant. The isolated compounds (syringic acid, silybin, kaempferol, apigenin, caffeic acid and genistein) were evaluated qualitatively and quantitatively. The study revealed that kaempferol has the highest concentration while genistein concentration was the least. The results obtained from *H. europaeum* study provide a good scientific base to examine the pharmacological effects of this plant in the future.

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