Phytochemical Investigation of Alkaloids in the Iraqi *Echinops heterophyllus* (*Compositae*)

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Abstract

Alkaloids are a group of naturally occurring chemical compounds that contain mostly basic nitrogen atoms . They are a large family of compounds synthesized by plants in addition to the bacteria, fungi, and animals, they often have pharmacological effects. The aim of this study is to isolate and identified alkaloids in a newly studied, wild Iraqi plant named *Echinops heterophyllus*. The medicinal importance of alkaloids, on one hand and the absence of any phytochemical investigation on heterophyllus species of echinops genus on the other hand , acquired this study its importance. Three alkaloids (named E1, E2 and E3) were isolated from seed plant part by two chromatographic methods: Preparative high Performance Liquid Chromatography (PHPLC) and preparative thin layer chromatography (PTLC), one of them identified as(1-Methyl-2,3-dihydro-4(1H)-quinolinone by different chemical analysis like: ultra violet spectrum analysis (UV spectrum), Fourier transforms infrared spectra (FT-IR) , elemental microanalysis (CHN) and Proton ^{1}H-NMR and carbon ^{13}C-NMR analysis.

Key words: Echinops, heterophyllus, quinoline alkaloids.

دراسة كيميائية للقلويدات في نبات شوك الجمل العراقي

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الخلاصة

القلويدات هي مجموعة من المركبات التي تصنع من قبل الكثير من النباتات وتلعب دورا مهما في معالجة و تنظيم الكثير من الامراض .الهدف من هذه الدراسة هو الفصل و التعرف على القلويدات الموجودة في نبات عراقي بري واسع الانتشار اسمه Echinops لم يدرس سابقا .
الاهمية الطبية للقلويدات و عدم وجود منشورات علمية تتناول المكونات الكيميائية لهذه النبتة اكسبت هذه الدراسة اهميتها .تم فصل ثلاث قلويدات من الاجزاء المختلفة من النبات و التعرف على التركيب الكيميائي لواحد منها بواسطة قياس (M.P, FT.IR, CHN, H^{1}NMR and C^{13} NMR)

الكلمات المفتاحية : القلويدات ، نبات شوك الجمل

Introduction

Echinops, a genus includes many plants which are individually referred to as globe thistle, is made up of more than 120 species of perennials, annuals, and biennials[(1,2)]. The genus belongs to the daisy family Asteraceae, and its species are found in Eastern and Southern Europe, Tropical and North Africa and Asia[(3).] In Iraq Ali Al-Rawi mention 11 species of *Echinops* in his book [(4)] among them *E. heterophyllus* P.H. Davis Family *(Compositae)* which was chosen for this study. This plant was wildly grown and widely distributed in Iraq specially in Erbil and Sulaimani. In Hanara village and surrounding area in Wadi Bastora and Shaklawa in Erbil governorate, the plant is called (Shakroka). The term (Shakroka) is come from that the circle-like part of the plant, before getting harderd in the late spring, is eaten and the taste is sweet, therefore, it is called shakroka:
Shakr means sugar , Shakroka sweet like sugar (oral communication) .

The *Echinopus heterophyllus* is a perennial, 40-100 cm high.Stems are simple or branching from the base, sparsely cobwebby-canescent. Leaves are lanceolate or oblong-lanceolate.[(5)] *Echinops* plant was reported to possess variety of compounds belonging to various classes like: alkaloids, flavonoids, terpenoids, lipids, steroids and polyacetylenes[(6)].

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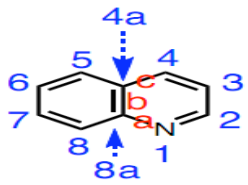
And many literatures survey revealed different pharmacological activities of *Echinops* plant like, antibacterial activity[7], Antifungal [8], Antioxidant activity [9], Protective effects on testosterone-induced prostatic hyperplasia [10], Hepatoprotective[11] and antiulcerogenic activity[12] .

All the alkaloids isolated from different species of *Echinops* related to the quinoline type so, the biosynthetic origin of quinoline alkaloids is the aromatic amine anthranilic (2-aminobenzoic) acid involved in the metabolism of the amino acid tryptophan. The skeleton of quinoline alkaloids constitutes a bicyclic system with a fused benzene and pyridine ring (figure 1). The attachment of a furan ring to the pyridine nucleus generates furoquinolines (e.g. furacridone), an important subgroup of quinoline alkaloids. The plant family *Rutaceae* represents the major source of quinoline alkaloids.Some of these naturally occurring quinolines have profound medicinal properties while others have served as lead



Quinoline
Benzo[b]pyridine
(1-azanaphtalene)

structures and provided inspiration for the design of synthetic quinolines as useful drugs[13].

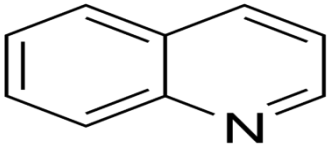


Figure 1: Basic structure of quinoline nucleus

This study was emphasized on the isolation and identification of alkaloids found in the Iraqi species of *Echinops* plant by two methods : Preparative high Performance Liquid Chromatography chromatography (PHPLC) and preparative thin layer chromatography(PTLC) and compare between quantity of these active constituents obtained by both methods.

Methods and Materials

Plant material

The whole plant of *Echinops heterophyllus* of the Family *(Compositae)* was collected from Nazali, 71Km north of Erbil . The plant was authenticated by Dr. Abdul-hussien Alkhait specialist in plant taxonomy .

The plant seeds were collected during the month of November (2011), while aerial parts (leaves/stem) and roots were collected during the months of May and June (flowering time) and were cleaned, dried at room temperature in the shade then pulverized by mechanical mills and weighed.

Thin layer chromatography was done on readymade plates of silica gel GF_{254nm} (20x20cm) of 0.25mm thickness (MERCK), using three different developing solvent systems:

S_1 = Benzene **:** methanol**:** (8 : 2)[14]

S_2 = Chloroform: acetone: diethylamine (5 : 4 :1)[14] .

S_3 = Toluene: ethylacetate: diethylamine (70 : 20 : 10)[15]

and detected by dragendorffs spraying reagent

Preparative HPLC was done using :- acetonitrile : water (65:35) as a mobile phase

Column: mediterranea C18 , 5 µm 15 X 2.12 cm, flow rate: 5 ml / min

Injection volume: 1 ml. Detection: UV. Detector at λ 205 nm.

Experimental work

The experimental work is divided into :

1- Preliminary phytochemical screening of various secondary metabolites like alkaloids, flavonoids, steroids, tannins, saponins, anthraquinioin, terpenoids and cardiac glycosides) in the different parts of *Echinops* plant.

2- Extraction of alkaloids.

3- Isolation and purification of alkaloids.

4- Identification and characterization of the isolated compounds.

Preliminary qualitative phytochemical analysis:

Chemical tests were carried out using the ethanolic extracts from plants and or the powdered specimens, using standard procedures to identify the active constituents.[16-18]

Test for alkaloids : Wagner's reagents **&** Mayer's

Test for flavonoids : Lead acetate test **&** NaOH test

Tests for steroids: Liebermann-Burchard test

Test for tannins : FeCl3 solution test

Test of saponins: foam test

Tests for anthraquinones: Borntrager's test

Test for terpenoids NaOH test

Test for cardiac glycoside: Keller-kiliani test

Extraction method

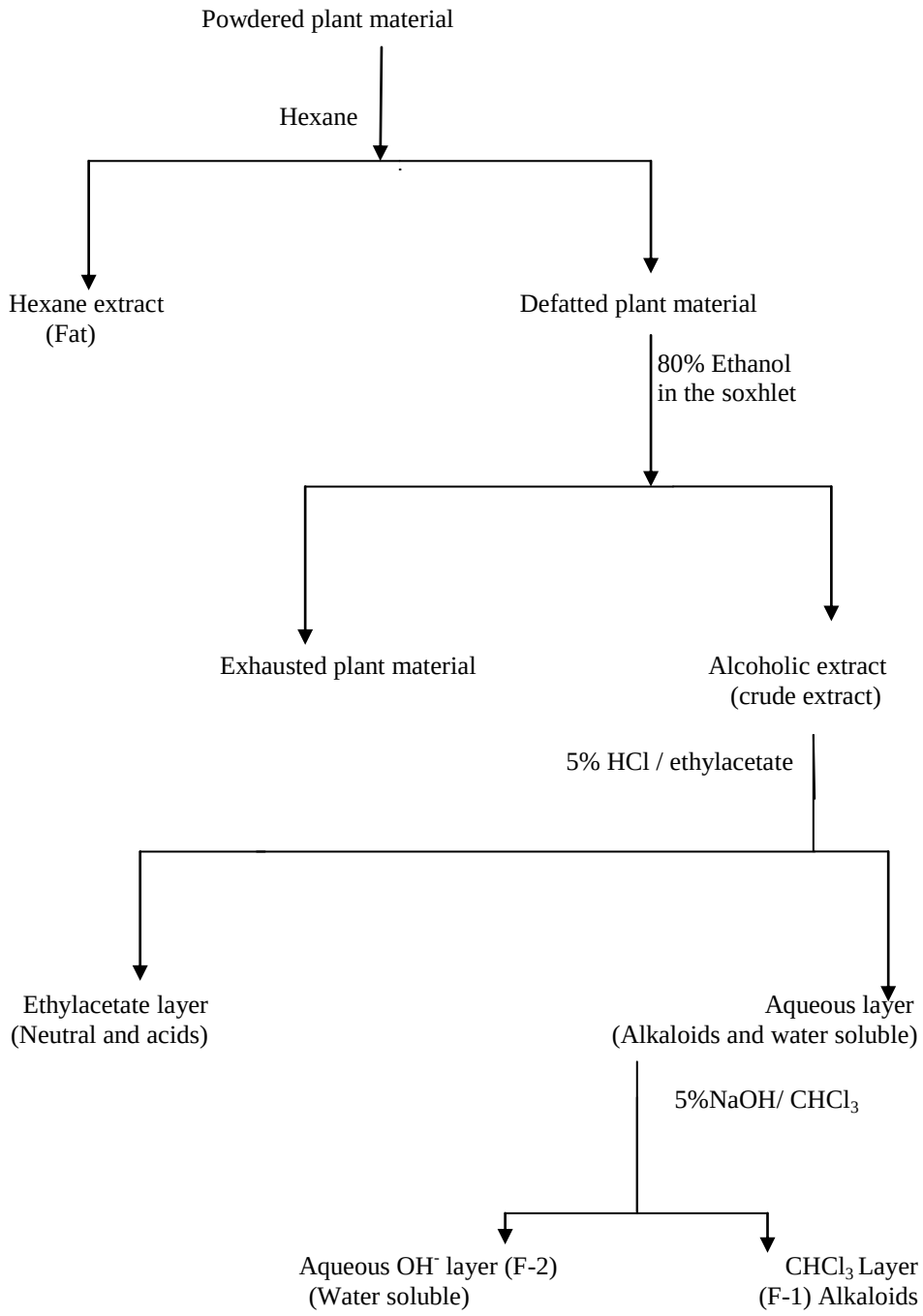


Figure:2 General scheme for separation of different plant constituents[17]

Results and Discussion

Preliminary qualitative phytochemical analysis:

The results of phytochemical screening are given in table (1).The results of preliminary phytochemical screening of plant extracts showed the presence of alkaloids, flavonoids, steroids, tannins and terpenoids in different parts of Iraqi species in different percentage, and the absence of, saponins, anthraquinoin and cardiac glycosides in all plant parts. These results can be compared with phytochemical screening of other *Echinops* species for example,: the aerial part of Iraqi heterophyllus species was contained traces amount of alkaloids, unlike Egyptian species *E. spinosissimus*, its aerial parts was contained about 11.3% alkaloids[19], also quinoline alkaloids and flavonoids found in the aerial part of Indian *E. echinatus* with the presence of tannins in the root parts only[20]. Many researchers reported that the concentration of secondary metabolites are varying from plant to plant belong to the same genus and even in the different parts of the same plant[21] , this is due to many factors like environmental heterogeneity, since the effect of environmental heterogeneity is highly scale-dependent. It may create high niche diversity and hence allow species to coexist at a large spatial scale[22], also the high complexity and heterogeneity of soil, like(soil structure, texture and depth, moisture retention characteristics, aeration) create a big variation in the chemical constituents even in the same country [23], good example seen in two Iraqi species of *Echinops* plant : *E. tenuisectus* and *E. heterophyllus*, phytochemical analysis of *E. tenuisectus* revealed the presence of high percentage of silymarine in the seeds (0.878%) and aerial parts (0.095)[24] with the absence of this compound in the *heterophyllus* species.

Table(1) Phytochemical screening of different parts of *Echinops heterophyllus*

Plant part	Al-kaloids	Flavo-noids	Stero-ids	Tann-ins	Sap-onin	Anthra-quinoin	Terpen-oids	Cardiac glycoside
Seeds	+	+	-	-	-	-	+	-
Aerial part	Traces	+	+	+	-	-	+	-
Roots	+	+	+	+	-	-	+	-

+, - represent presence and absence of phytoconstituents respectively.

Preliminary identification of different Echinops parts by TLC

Thin layer chromatography of fraction 1 (F- 1) obtained from different parts of the *Echinops*, confirms the following:

(a)The presence of three different alkaloids in fraction-1 (named E1, E2 and E3) which is obtained from seeds part and two alkaloids in the same fraction obtained from roots part (E1 and E2) with very traces one compound (E1) in the alkaloidal fraction of aerial plant . figure-3The R_f values of these compounds in the different solvent systems were calculated, table(2).

Table (2) R_f values of alkaloids obtained from different plant parts in different developing solvent systems in TLC.

Compound	Plant part	S_1	S_2	S_3
E1	Seed	0.16	.22	0.25
E2	Seed	0.58	0.68	0.66
E3	Seed	0.75	0.8	0.79
E1	Root	0.17	0.25	0.26
E2	Root	0.6	0.7	0.67
E1	Aerial part	0.15	0.21	0.25

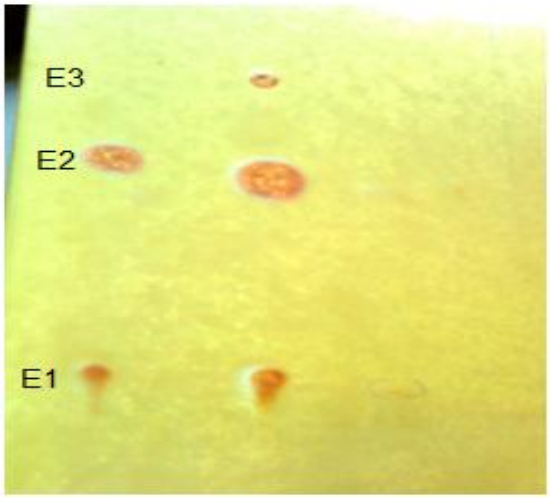


Figure 3: TLC of fraction one (F-1) for different *Echinops* parts.

(roots, seeds, aerial parts) using silica gel GF_{254nm} as adsorbent and S_1 as a mobile phase. Detection by dragendorffs spraying reagent

R : Roots S : Seeds A : Aerial part

Isolation and purification of alkaloids:

Two chromatographic analysis were carried out to isolate in a pure form three alkaloids (named E1, E2, E3) found in the plant which are:

Preparative HPLC and preparative TLC, since seeds contain the largest number and highest quantity of the alkaloids so alkaloids fraction obtained from seeds part was used to separate and isolate these compounds in a pure form.

Isolation and purification of alkaloids by preparative HPLC

One gram (1 gm) of F-1 obtained from plant seeds dissolved in a minimum quantity of chloroform was injected in to preparative HPLC

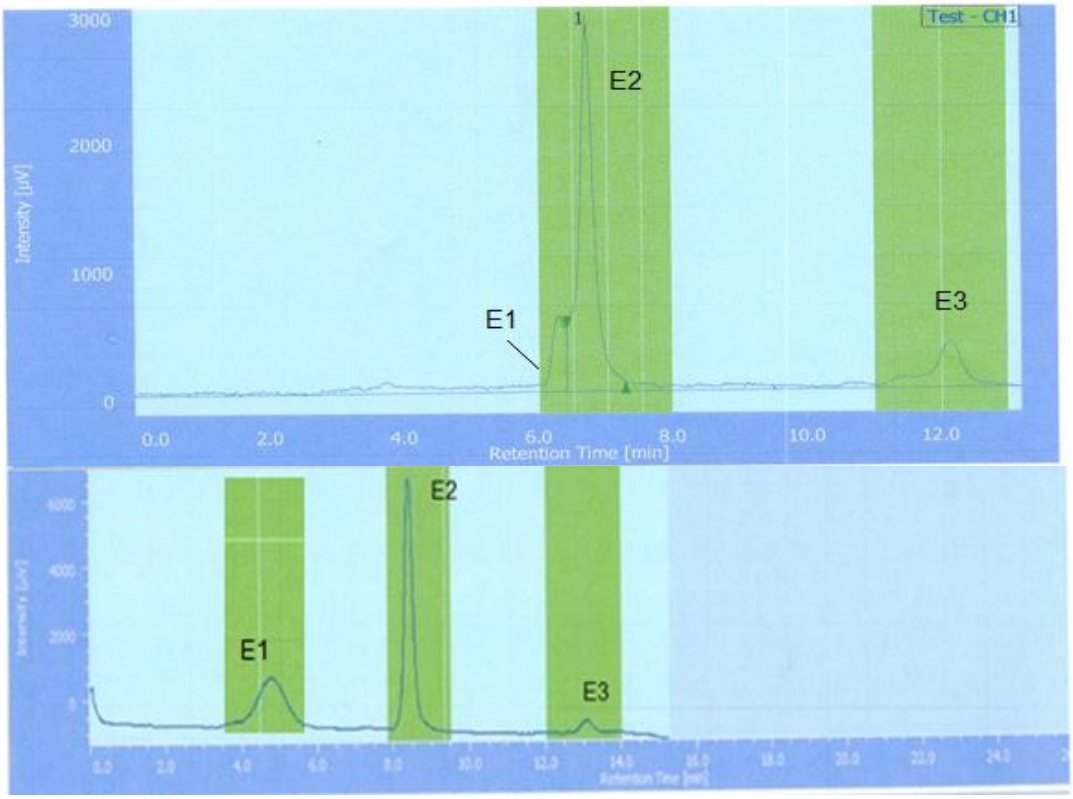


Figure 4: Preparative HPLC analysis of fraction-1 obtained from seeds plant observing three peaks represent three different compounds, one of them (E2) is a major one.

Three samples obtained from preparative HPLC were weighted and subjected to co-TLC .Weight of E1 = 0.07 gm , weight of E2 = 0.5 gm, weight of E3 = 0.16 g

Isolation and purification of alkaloids by preparative TLC

On a 20cm x 20cm glass plates a slurry of 75 gm of silica gel GF 254 suspended in 150 ml of distilled water was applied in 1mm thickness manually by using Jobling laboratory division plate coater. The freshly coated plates were left until the transparency of the layer disappears. After 10 minutes, the plates stacked in a dry rack and heated in vertical position for 1 hour at 110^{o}C with occasional opening of the oven door from time to time in order to allow moisture escape. The completely dried and activated plates were kept in a dry and moisture free container containing adsorbent silica gel

One gram (1 gm) of F-1 obtained from plant seeds (highest quantity) dissolved in a minimum quantity of chloroform and applied on a number of preparative TLC plates using S_{1a} solvent system. The solvent was allowed to rise to a height of 15cm from the base line. One major and two minor bands were observed after spraying a side of plates with dragendorffs three band had been scrapped off, eluted with chloroform, then filtered. The filtrate evaporated to dryness, in vacuo to give white crystals, upon re-crystallization out of boiling ethylacetate, a fluffy white crystals of E1, E2 and E3 were obtained.

Three samples obtained from preparative TLC were weighted and subjected to co-TLC

.

Weight of E1 = 0.037 gm , weight of E2 = 0.327 gm, weight of E3 = 0. 063 gm

From the above results, the quantity of compounds obtained in a (pure form) by Preparative HPLC is higher than that obtained by preparative TLC. Classical preparative TLC suffers from several drawbacks, the main disadvantage being the removal of purified substance from the plate and its subsequent extraction from the sorbent, other drawbacks include the length of time required for the separation and degree of purity for the separated compounds [(25)], compare with preparative HPLC, which is consider know, the most powerful and versatile method for purification tasks in the pharmaceutical industry[(26)]. Despite the fact that among the tools used in the large scale purification of pharmaceuticals, Preparative HPLC is one of the more expensive and solvent-consuming approaches, it yields the highest-purity drug substance. The interest in preparative HPLC will continue to grow because of the increasing uncertainty in the market expectations for product purity. Its nearly linear scalability makes preparative HPLC one of the more viable approaches to compound purification[(27)].

Characterization and Identific -Ation of The Isolated Alkaloids (E2):

1- Melting point:-

The isolated compound which is named E2 had a sharp melting point of 160-162˚C

2- Ultra violate spectra:

The isolated alkaloid (E2) show UV absorption near 242nm

3- FT-IR spectra:

The identification of the unknown alkaloid (E2) was further confirmed by using FT-IR spectroscopy figure (5) . The characteristic IR absorption bands showed by this compound are listed in table(3).

Table (3)Characteristic IR absorption bands(in cm^{-1}) of the isolated alkaloids[(28)]

Functional group	Group frequency wave number (in cm^{-1})	Assignment
—N<	3306, 3245	N–H stretch (two band for tertiary amine
C-H	2910-2852	Asymmetric and symmetric stretching of CH_3
C=O	1590-1750	C=O stretching vibration (conjugated)
C-N	1333,1336	C-N stretching bands of tertiary amine
CH_3	1430,1480	C-H bending vibration
C-H	914, 868, 750	C-H of aromatic group out of plane

4- Elemental micro analysis (CHN):

Elemental microanalysis was performed for unknown isolated compounds (E2) to confirm their chemical structure. The result of this analysis (table 5) showed that the unknown compounds consist of carbon , hydrogen, oxygen and nitrogen in different percentage.

Table(4) Elemental microanalysis of the unknown isolated alkaloid

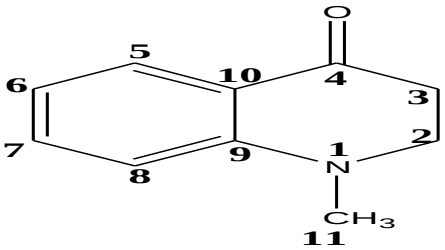
Name	C%	H%	O%	N%
E2	74.07	6.208	10.25	9.463

5- H^1 and C^{13} NMR

The E2 compounds presented ^{13}C NMR spectra (DMSO, 75 MHz): with chemical shifts typical of quinoline rings[(28)] in the ranges of *δC* 21.12 (C-2), 24.77 (C-3), 170.12(C-4),126.987 (C-5), 121.825 (C-6), 127.640 (C-7), 114.951 (C-8), 138.26 (C-9), 123.47 (C-10), 30.4 (C-11).

^1H NMR (DMSO-d6-, 300 MHz) revealed that E2 compound undergo tautomerism which lead to the appearance of chemical shifts of the hydroxyl group at 10.02 at (C-4), 2.4 (3H, as a singlet of the methyl protons), 2.6 (2H, *d*, H-2),5.09 (1H,s, H-3), 6.84-7.15 (4H, *m*, H-5 ,H-6 , H-7 , H-8). Figure (7).

Depending on the above results, the expected chemical structure for the isolated E2 compound is:



1-Methyl-2,3-dihydro-4(1H)-quinolinone,

It is a new compound isolated (for the first time) from Iraqi ***Echinops heterophyllus*** plant, it seen to be the hydrogenated form of echinopsine (1-Methyl-4(1H)-quinolinone), an alkaloid isolated from 14 species of *Echinops* plant.

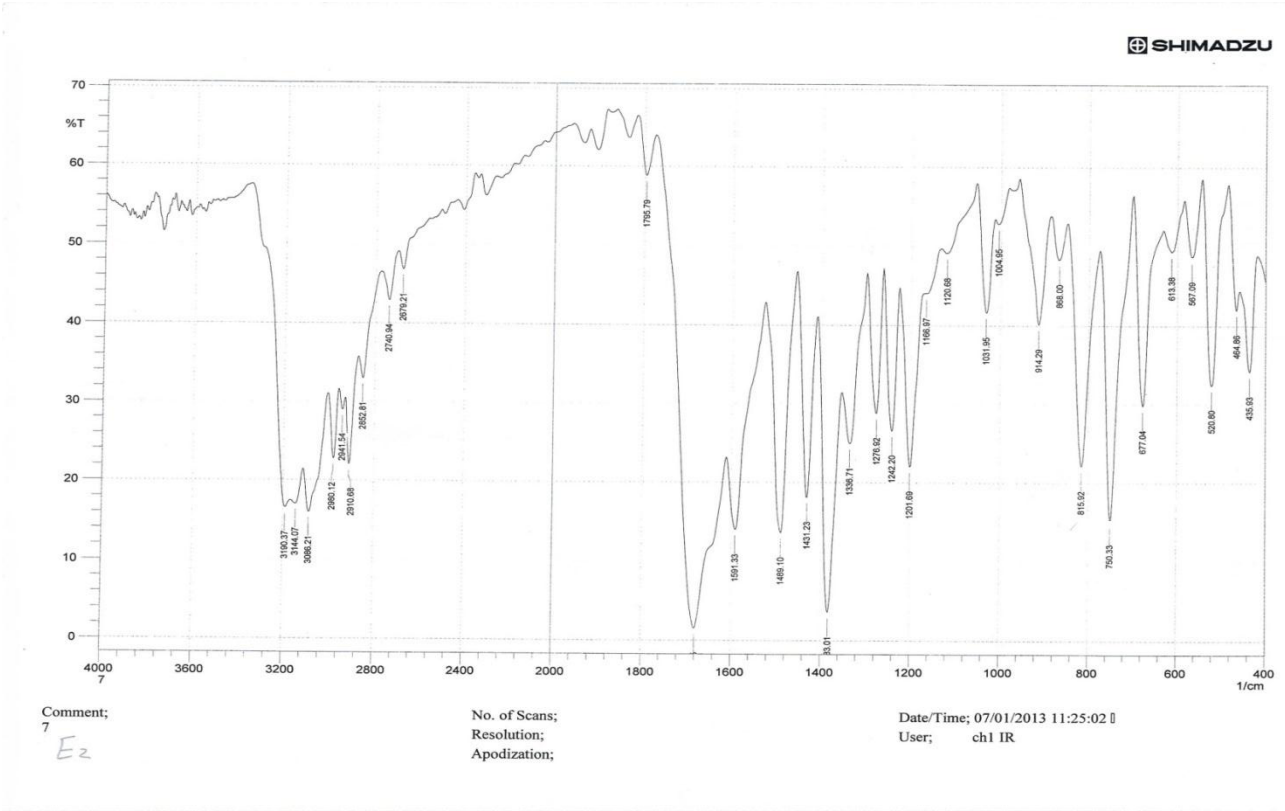


Figure 5: FT-IR spectrum of the isolated alkaloid (E2)

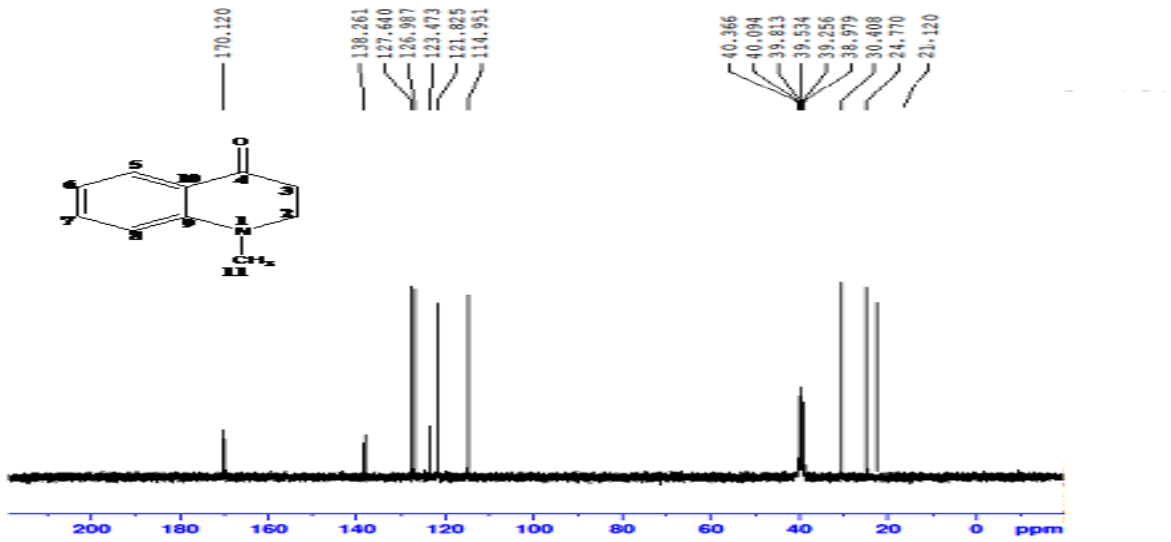


Figure 6 : ^{13}C-NMR analysis of the isolated E2 compound

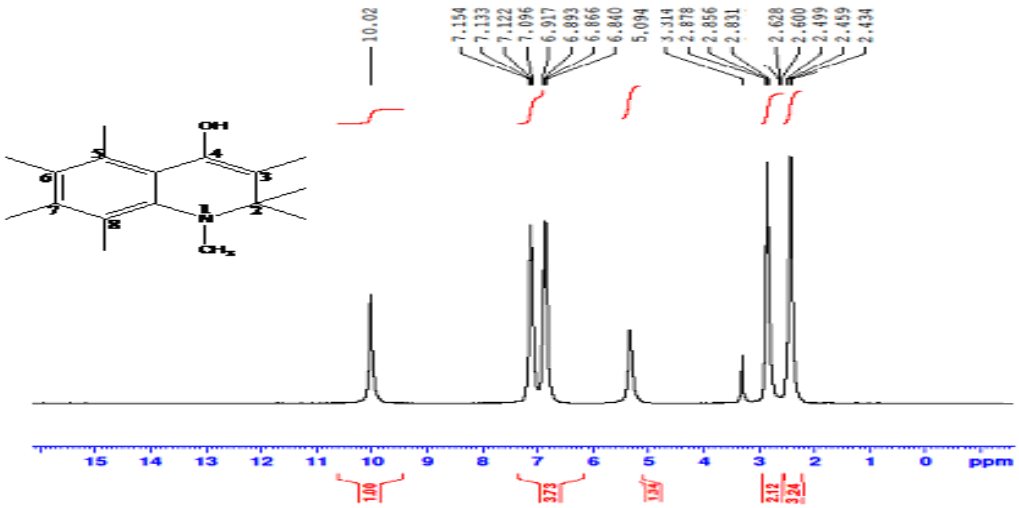


Figure 7 : ^{1}H-NMR analysis of the isolated E2 compound.

Conclusion

Phytochemical investigation of a new wild Iraqi plant used traditionally for wound healing and snake bit named ***Echinops heterophyllus*** was done and the results revealed the presence of alkaloids, flavonoids, terpenoids, tannins and steroids in the different plant parts and in a different percentages, aerial parts contain the highest quantity of flavonoids, while seeds contain the highest amount of alkaloids. General schematic procedure of Jeffirey B. Harborne was used to extract different plant parts using 80% ethanol in soxhlet apparatus.Two chromatographic analysis were carried out to isolate in a pure form three alkaloids from seeds part (which contain highest quantity) : preparative HPLC and preparative TLC, where the quantity of compounds obtained by preparative HPLC was higher than that isolated by preparative TLC.

The benefit of preparative HPLC to isolate the maximum amount of desirable products at a desired purity in a minimum of time from different Iraqi medicinal plants to use it as a standard reference or as lead structures for the design of useful drugs in the future studies .Preparative HPLC can be used in pharmaceutical development for troubleshooting purposes or as part of a systematic scale-up process.

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