Qualitative and Quantitative investigations of Furocoumarin derivatives (Psoralens) of *Haplophyllum tuberculatum* (Rutaceae)

Arif Diar
Jawad Enas*
Al-Khateeb Ekbal
Al-Shamma Ali

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الخلاصة

أن نبات الهيبولوفيلم من النباتات الطبية الموجودة في العراق والذي ينمو بصورة طبيعية في أماكن متعددة ويعرف بأسم الزفرة أو جويفة ويستخدم من قبل الأهالي كطارد للأرياح وخاصة في حالات المغص المعوي للأطفال .
لقد أثبتت دراسات عديدة بأن هذه النبتة غنية بأشباه القلويات والزيوت الطيارة ولكن لا توجد أي دراسة تتناول هذه النبتة من ناحية أحتوائها على مواد الفيروكومارين (Psoralen) لذا وجد من الضروري دراسة هذه المكونات وفصلها بالطرق المتبعة.
أن النتائج الأولية أثبتت لنا بأن النبتة تحتوي على مواد الفيروكومارين (Psoralen)، لذا فقد تم أستخلاص هذه المواد باستعمال البتروليوم ايثر الذي يغلي بدرجة حرارة ما بين 60-80° م كمذيب عضوي .ولقد أثبتت لنا نتائج فحص المستخلص بطريقة كروماتوغرافيا الطبقة الرقيقة عن وجود مركب moidin الذي تم فصله عن بقية المركبات الموجودة في هذه النبتة على شكل بلورات بيضاء خفيفة وطويلة وذات درجة حرارة أنصهار تبلغ 148° م.
أخذت عينات مختلفة من نبات الهيبولوفيلم من عدة مناطق من العراق من العظيم في محافظة ديالى وداقوق في محافظة التأميم ومن قرية العوينات في محافظة صلاح الدين , وتم أخذ العينات في فترات مختلفة من أشهر السنة. وتبين من الفحص الأولي أن مادة الأمويدين تزداد نسبياً مع تقدم الوقت حيث تكون في ذروتها في شهري كانون الأول وكانون الثاني.

ABSTRACT

Haplophyllum tuberculatum is an indigenous plant widely distributed in Iraq.
Phytochemical investigation of this plant indicating that the plant is rich in alkaloids, fixed and volatile oils; but non about psoralen contents. Preliminary investigation indicated that this plant has furocoumarins in acceptable amount.
Based on these results together with literature survey it was deemed desirable to carry out this phytochemical work with emphasis on isolation and characterization of the furocoumarins compounds.
Detailed preliminary investigation of the furocoumarins content of the dried, ground whole plant has been described and the results were discussed.
Thin layer chromatography of the petroleum ether (boiling point 60-80ºC) extract demonstrated the occurance of furocoumarins compounds in the plant.
The extraction procedure of the plant material, the fractionation and isolation procedures of the furocoumarins are fully described.
One compound was isolated and identified as Ammoidin by TLC (compared with standard), melting point (148°C), mixed melting point (148ºC) and HPLC.
Spectrophotometer apparatus was used to determine exactly the amount of Ammoidin measured at 304nm at UV absorption. In Haplophyllum tuberculatum the maximum amount of Ammoidin was found in sample collecting from Daquok during June.

* Department of Pharmacognosy, College of Pharmacy, Baghdad University, Baghdad – Iraq

INTRODUCTION :

Haplophyllum tuberculatum Forssk Adr.Juss (Rutaceae) is an indigenous plant widely distributed in Iraq[1], it is also known as wild Rue, ZIFRA or JUWAIFA. In Iraq the Haplophyllum.tuberculatum grows all over the country, in the northern parts like Kirkuk and Tuzkhurmat, in the central parts including Khanaquin, Mandeili, Khalis, Fallujah while in the south it grows near Basra and Maisan[2].Literature survey realed that a number of phytochemical investigation studies have been reported indicating that the plant is rich in alkaloids[3], fixed and volatile oil[4,5] but non about furocoumarin (Psoralen) contents. Since this plant is a member of rutaceae family,a family reported to be rich in furocoumarin derivatives our attention was directed toward the identification of these compounds in this plant. These compounds are of economical value as they are used in dermatological preparation for different skin diseases. They have been prominent in the United States for their role in photochemotherapy (PUVA) of vitiligo[6-8], psoriasis[9-11], Parapsoriasis, Mycosis fungoides[12-14]. Recently some other biological activities of Psoralens and related compounds have been reported; these include: having anti-inflammatory and analgesic activities[15], antitumor activity[16], having calcium antagonist properties[17]. Also these compounds show encouraging levels of phototoxicity against fusarium culmorum[18] and may have a potential use as photoactive pesticides.

EXPERIMENTAL:

Plant material :

Different samples of Haplophyllum tuberculatum were collected from different locations at different times.
All samples authenticated by National Iraqi Herbarium, Botany Directorate at Abu-Ghraib.

Extraction:

Four hundred grams of moderately coarse powder of Haplophyllum tuberculatum (aerial part) was extracted with (2.5 liter) of petroleum ether (b.p. 60-80ºC) using flask fitted with a reflux condenser, heated for three hours. The extract then filtered with suction and the marc was re-extracted several times until complete exhaustion. The extracts were combined and allowed to separate from the mother liquor. This resinous material was chromatogram indicated the presence of alkaloids (spraying with Dragendroff's reagent) in addition to the psoralen derivative.
The greenish resinous residue was dissolved in 25ml of 5% sodium hydroxide solution and partitioned with 25ml of chloroform in a separatory funnel and left for 15 minutes. Two layers were observed, the upper layer (the aqueous alkali layer) and the lower layer (chloroform layer). The upper layer was separated, neutralized with 5% HCL solution, and extracted with chloroform.
The chloroform solution was evaporated to dryness to leave a residue of (0.15). TLC of this residue showed the presence of psoralen derivatives together with some traces of the alkaloid γ-fagarine, further purification of furocoumarin (psoralen) derivatives by preparative thin layer chromatography (TLC).
It was found that plant material (Marc) left after petroleum ether extraction contained a good quantity of psoralen so it was dried thoroughly and re-extracted to be determined quantitatively twice with 100ml. of ethanol each time for 3 hours, or until complete exhaustion. The ethanol extracts then were combined together and evaporated to dryness under reduced pressure at a temperature not exceeding 40°C to give orange residue[19]. (Fig.1).

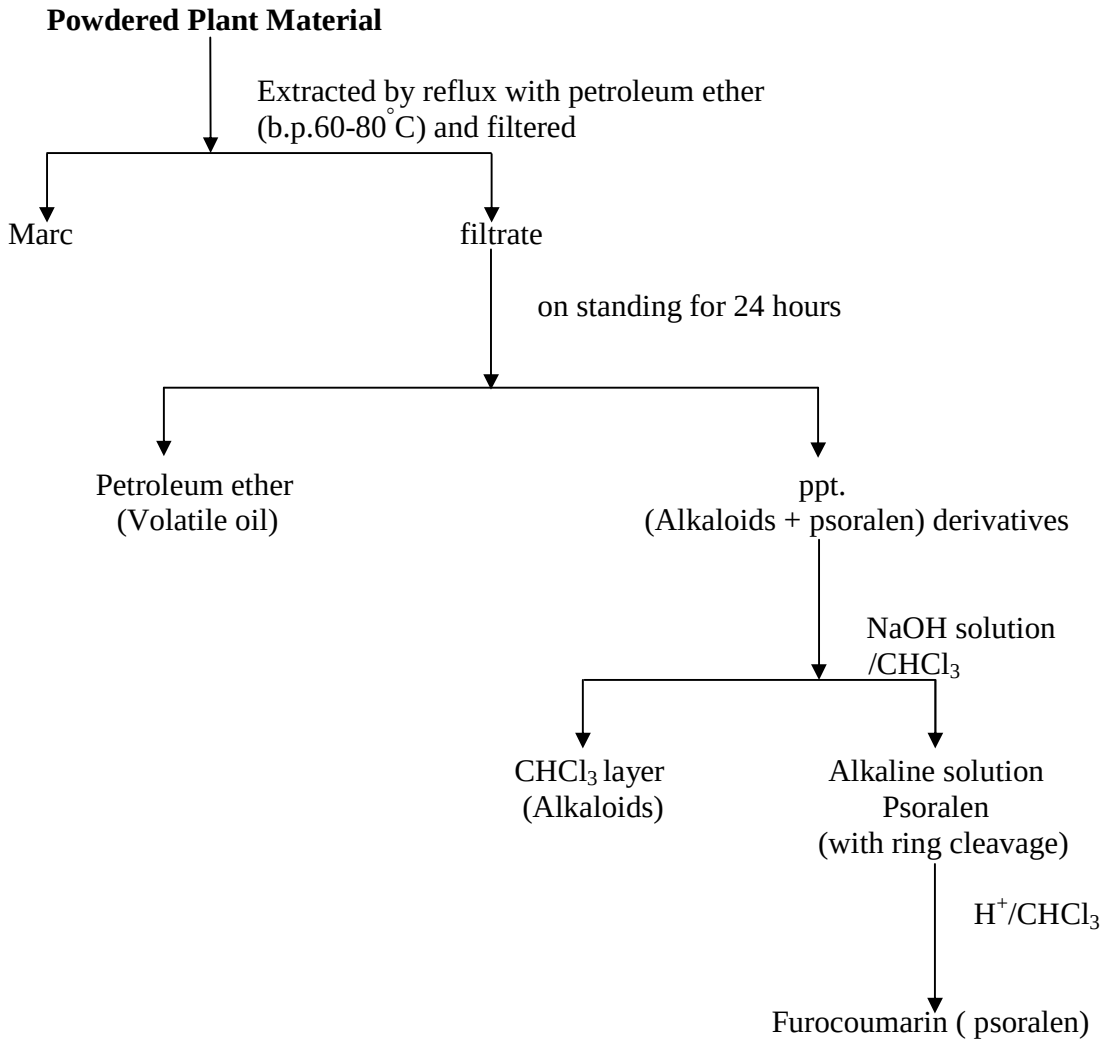


FIG 1 . SCHEMATIC PROCEDURE FOR THE EXTRACTION AND SEPARATION METHOD OF FUROCOUMARIN (PSORALEN)FROM HAPLOPHYLLUM TUBERCULATUM.

RESULTS

The identification of furocoumarin (psoralen) derivatives (Ammoidin) was approved by TLC using the following system:
Silicagel GF254 as a stationary phase with two solvent systems: Benzene:Acetone (90:10 v/v) and Chloroform:Methanol (97:3 v/v) and standard reference.
The identified compound was further approved by HPLC as shown in (Fig-2,3,4).
Potentiation of the isolated ammoidin together with the standard compound (Fig-3,4) show a sharp peak with higher intensity.

Condition of the HPLC
Schimadzu: 6A
Column: C18 25cm.
Solvent: 95% ethanol
Wave length: 254nm
Flow rate: 1 ml/min
Mobile phase: (1) 50% Acetonitril in water
(2) 65% Methanol in water

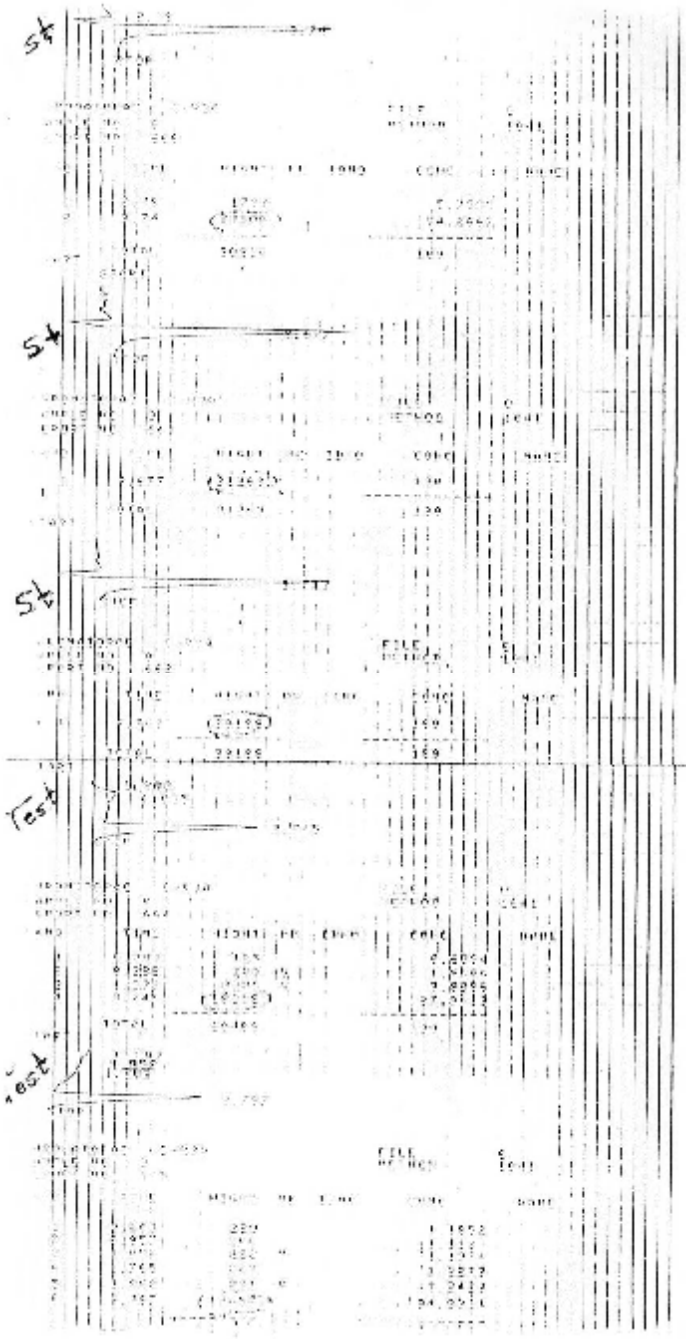


FIG 2 . HPLC FOR THE SEPARATED COMPOUND (AMMOIDIN) COMPARED TO THE STANDARD REFERENCE .

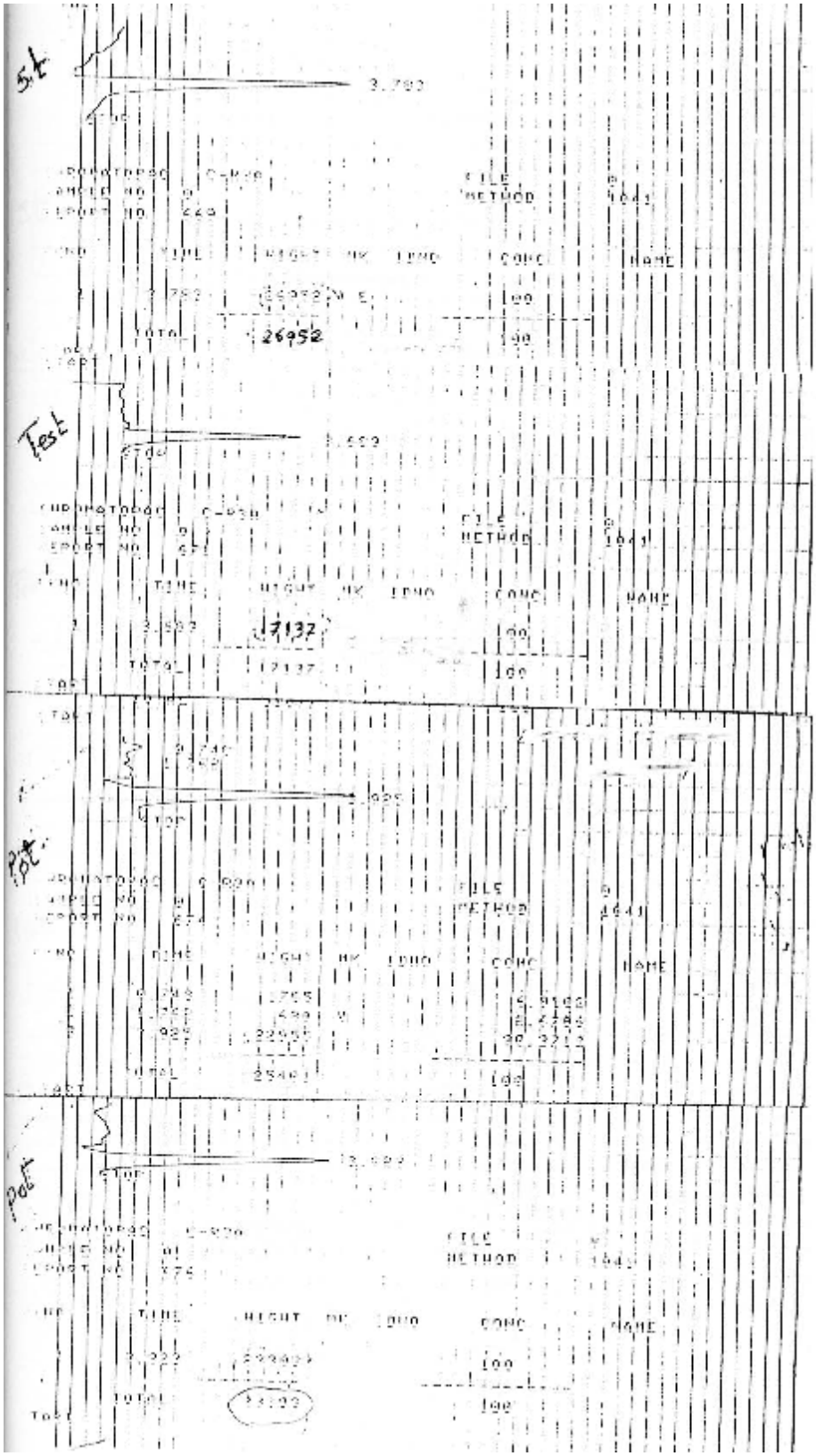


FIG 3 . HPLC FOR A MIXTURE OF EQUAL QUANTITIES OF THE SEPARATED COMPOUND (AMMOIDIN) AND THE STANDARD REFERENCE WITH THE FIRST MOBILE PHASE .

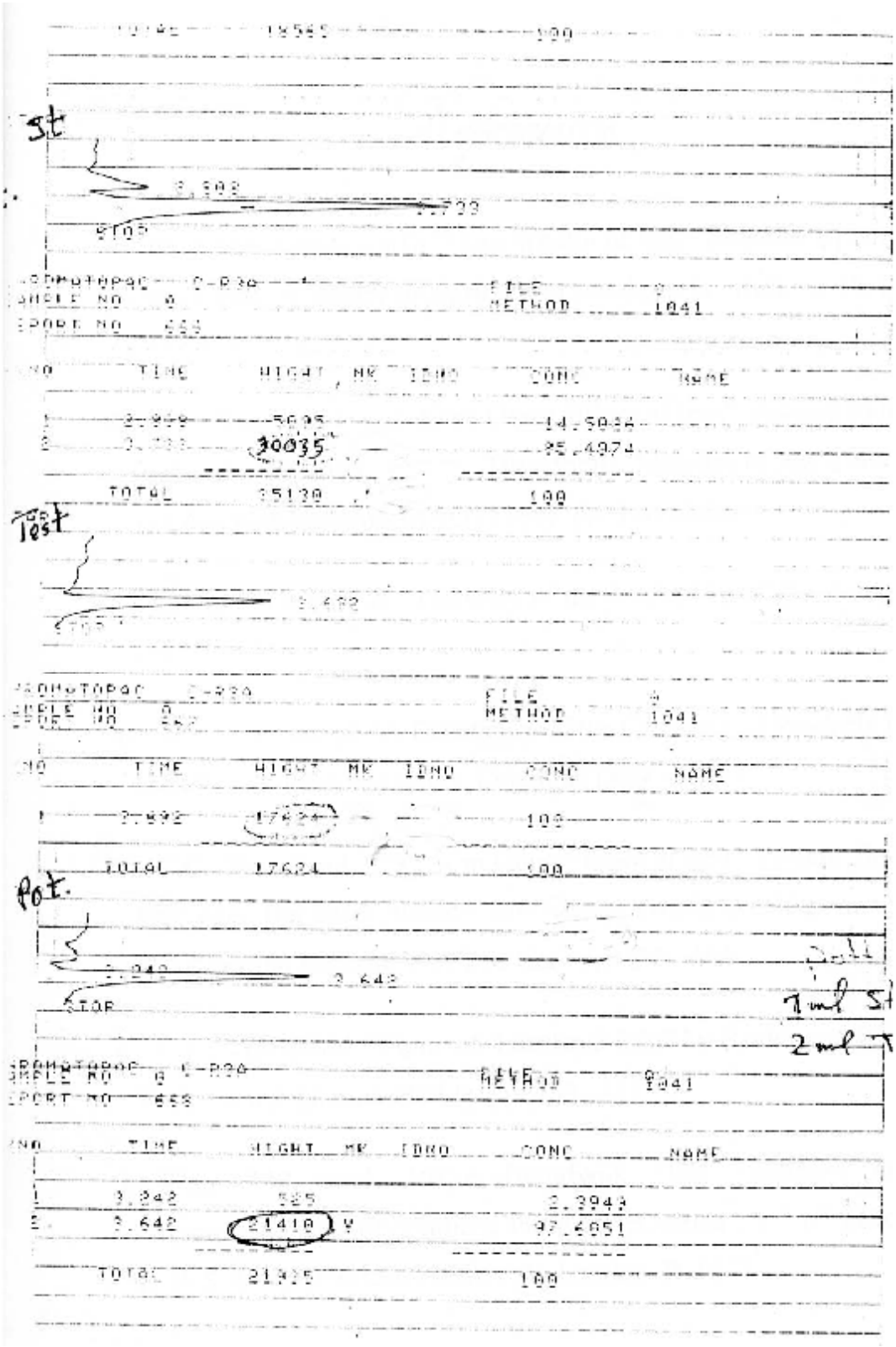
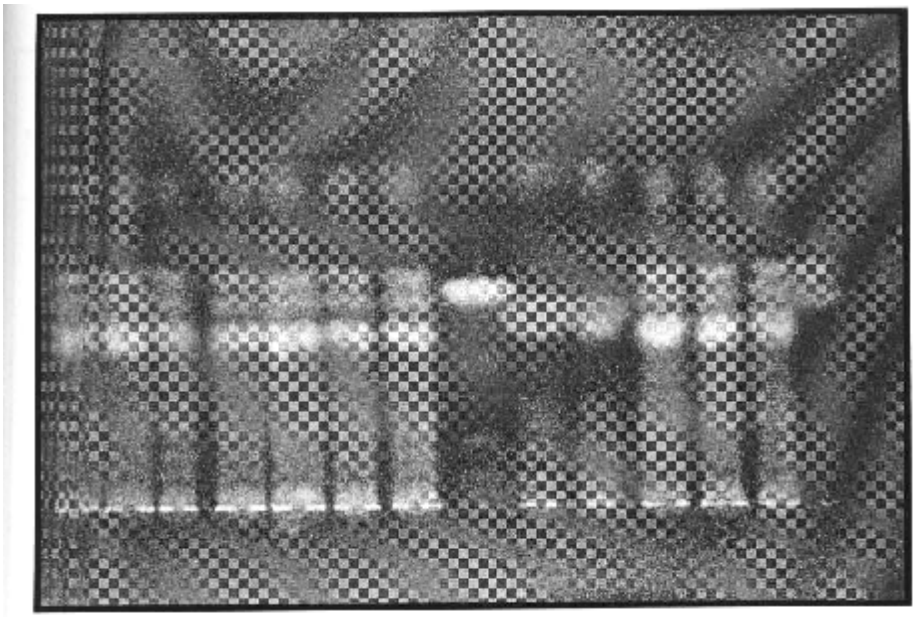
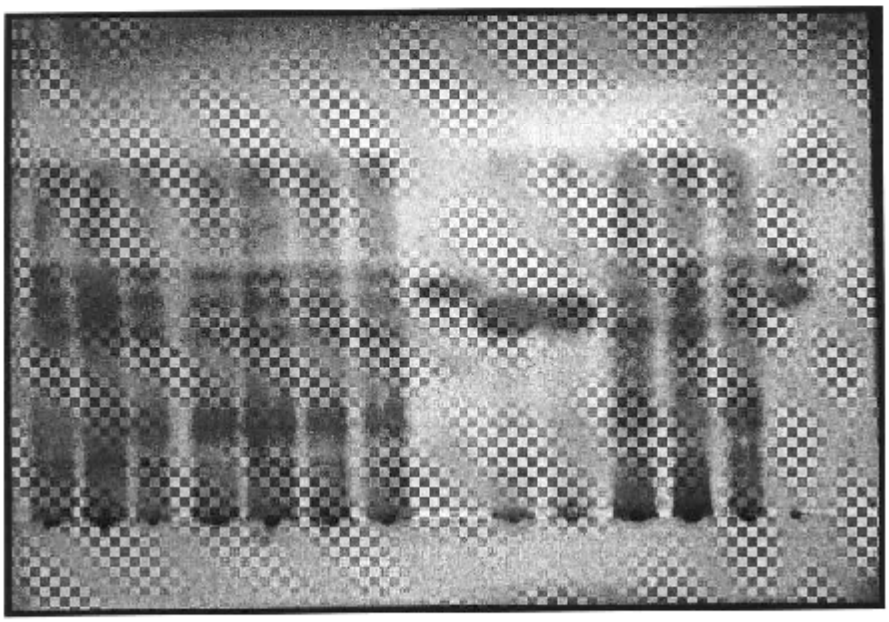


FIG 4 . HPLC FOR A MIXTURE OF EQUAL QUANTITIES OF THE SEPARATED COMPOUND (AMMOIDIN) AND THE STANDARD REFERENCE WITH THE SECOND MOBILE PHASE .

Further, the compound approved by spectrophotometer as shown in Figs.5 and 6.



UV light 366



UV light 254

Fig -5 -6-

Represent TLC of Haplophylum tuberculatum obtained from petroleum ether extract the aerial part, whole plant, and root and the petroleum ether collecting at different times together with standard reference St.(2) and St.(3), using solvent system No. 2 and silica gel GF.
The chromatogram was visualized by UV 366 nm.

The standard solution of Methoxsalen was separated from Neomeladinin tablet in pure form, by triturating with hot ethanol several times. The ethanolic solutions then collected together and evaporated to dryness to leave white powder, recrystallized out of ethanol to give white to greenish crystals, M.P. 146-148ºC[20].
100mg. of methoxsalen was dissolved in methanol and the volume was adjusted to 100ml. using volumetric flask and this was referred to as a stock solution[21].
Methanol was used to give zero point absorption at wave length 304nm. Different dilutions of the stock solution were measured to give certain absorption readings. The absorbance verses the concentration gave a straight line as shown in Fig.-7.

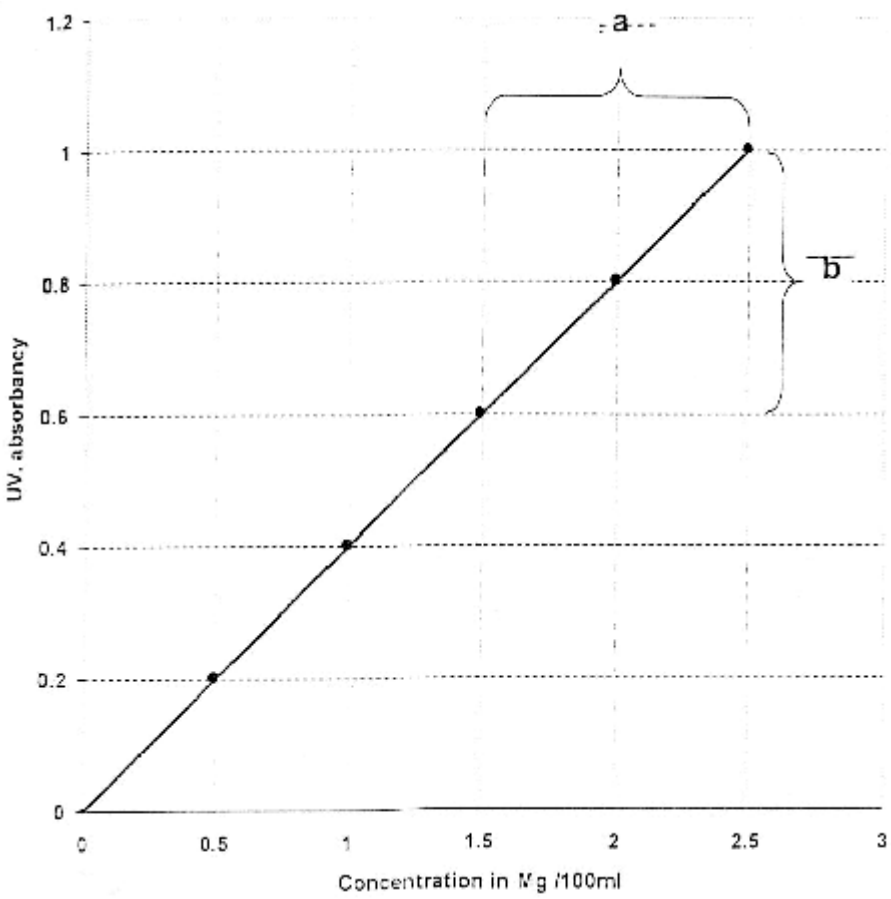


FIG 7 . STANDARD CURVE OF AMMOIDIN (METHOXSALEN) AT WAVELENGTH 304 NM

a: any point on Y axis
b: any point on X axis

slope = a/b = 1.5/0.6 = 2.5 ---------- (22)
or = (2.5-1.5) / (1-0.6) = 2.5

1/slope = Calibration factor (correction factor) ---------- (22)
= 1 / 2.5 = 0.4

Absorption * calibration factor = concentration

A * C.F = C ---------- (23)

Concentration * Volume = amount
C.V. = amount ---------- (23)

The work was continued to determine this content quantitatively, by spectrophotometric method. The study was carried on, by collecting different samples of the plant from different locations and different times (Table 1,2,3) and (Fig. 8).

Table 1 . Represent the absorbency and the amount of Ammoidin (methoxsalen) in µg/100gm (calculated according to the standard curve) of the petroleum ether extract residue of Haplophyllum tuberculatum collected from different localities at different times.

H. tuberculatum collected at	Absor.	Volume	Conce.	Amount /25 gm	Amount /100gm
April	0.684	1000	0.2736	273.6	1094.4
May	1.206	1000	0.4824	482.4	1929.6
June	3.733	1000	1.4932	1493.2	5972.8
August	1.896	1000	0.7584	758.4	3033.6
May*	2.024	1000	0.8096	809.6	3238.4

* May from Salah Aldin

Table 2 . Represent the amount in µg/100gm of Ammoidin (methoxsalen) with absorbency (calculated according to the standard curve) for all samples of Haplophyllum tuberculatum obtained out of the ethanolic extracts .

H. tuberculatum collecting at	Absor.	Volume	Conce.	Amount in 25gm	Amount /100gm
April	2.584	1000	1.0336	1033.6	4134.4
May	1.713	2000	0.6852	1370.4	5481.6
June	3.000	1000	1.2	1200	4800
August	2.511	1000	1.0044	1004.4	4017.6
May*	2.897	1000	1.1588	1158.8	4635.2

* May from Salah Aldin

Table 3 . Comparative amounts of Ammoidin (methoxsalen) calculated out of petroleum Ether residues and ethanolic residues, in µg/100gm collected from Different localities at different times of Haplophyllum tuberculatum .

H. tuberculatum	April	May	June	August	May*
methoxsalen	5228.8	7411.2	10772.8	7051.2	7873.6

* May from Salah Aldin

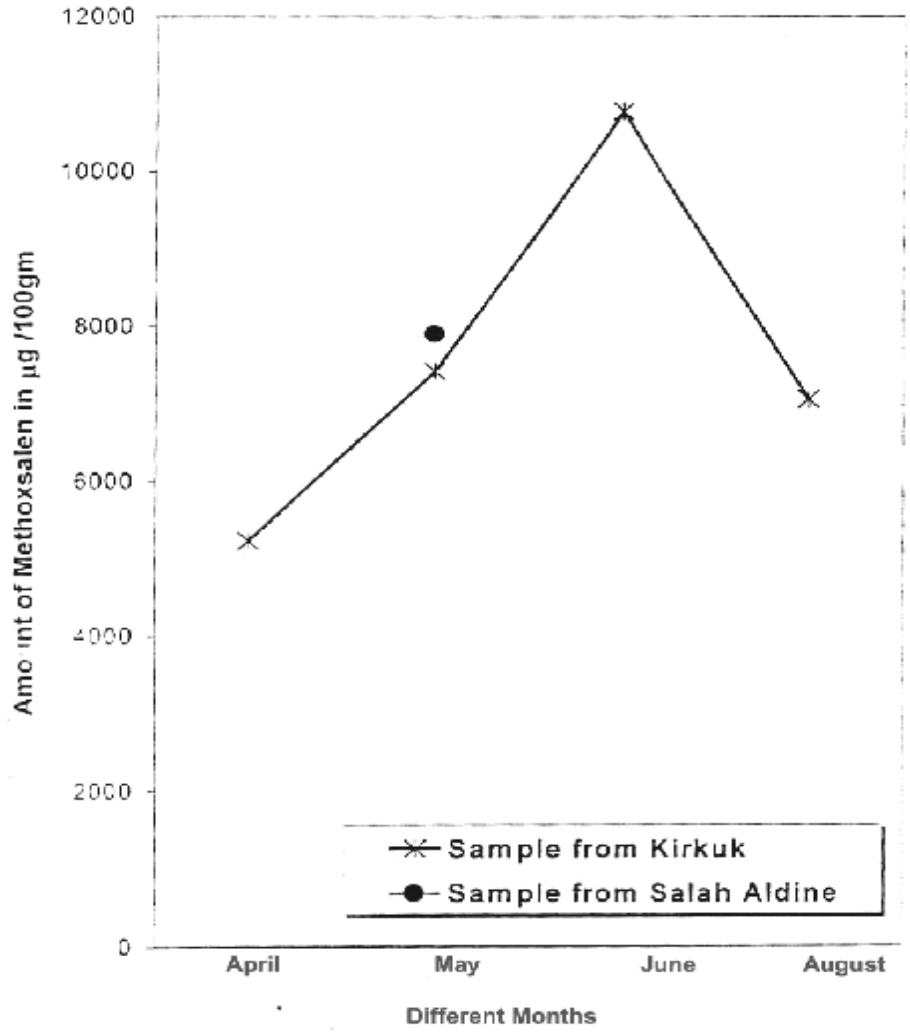


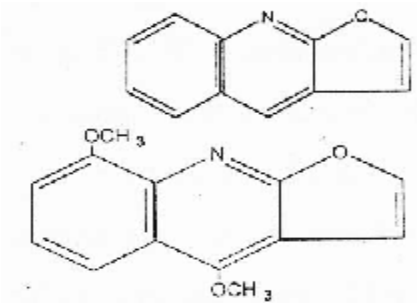
FIG 8 . COMPARATIVE AMOUNTS OF METHOXSALEN CALCULATED OUT OF PETROLEUM ETHER RESIDUES AND THANOLIC RESIDUES, IN µG/100GM OF HAPLOPHYLLUM TUBERCULATUM COLLECTED FROM DIFFERENT LOCATIONS AT DIFFERENT TIMES

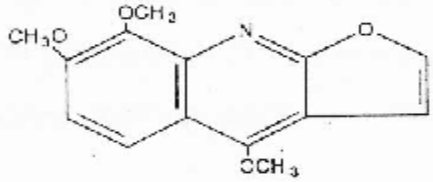
Three different samples were collected from Al-Ethaim area, 170-180km. north of Baghdad. The first one, was on April 17th, 2001, the second was on May 16th, 2001, and the third one was on June 2th, 2001. One sample was collected from Al-Auwainate area, about 10-15km. south of Tikrit on May 9th, 2001.
More samples were collected from Daquok area, about 60km. south of Kirkuk, The first one was collected on June 28th, 2001. Other samples were collected during August 2001.

DISCUSSION:

Furocoumarin (psoralen) compounds are considered as one of the important class of natural compounds, which are widely used to treat dermatological diseases. The available imported preparations of these compounds are very expensive.
Recently, our Pharmacognosy Department interested and engaged in a program, searching for plant sources rich in psoralen derivatives.
Rutaccae, as reported in the literature, is a family rich in these compounds.
Since Haplophyllum tuberculatum is a member of this family, and it is wildly and widely distributed in our country, we found it is worth while to study this plant and investigate its furocoumarin (psoralen) content. Previous phytochemical studies of this plant from our Pharmacognosy Department covered the alkaloids[3], the volatile oils[5], and the fixed oil contents[4].
Preliminary investigation of the extract of this plant by TLC indicated the presence of furocoumarin (psoralen) derivatives, mainly ammoidin. The separation of these compounds was carried on by extraction of the plant material with petroleum ether. TLC of this extract indicated the presence of furocoumarin (psoralen) derivatives together with some of the alkaloidal content like skimmianin and γ- Fagarine.

These alkaloids are furoquinoline derivatives

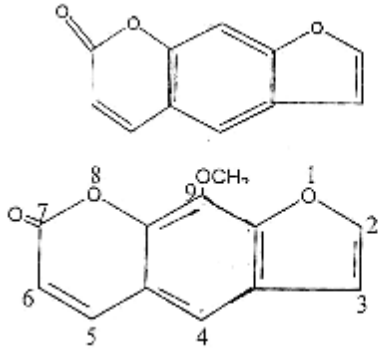




γ – Fagarine
4,8-Dimethoxy-furo [2,3-b] quinoline

Skimmianine
4,7,8-Trimethoxy-furo [2,3-b] quinoline

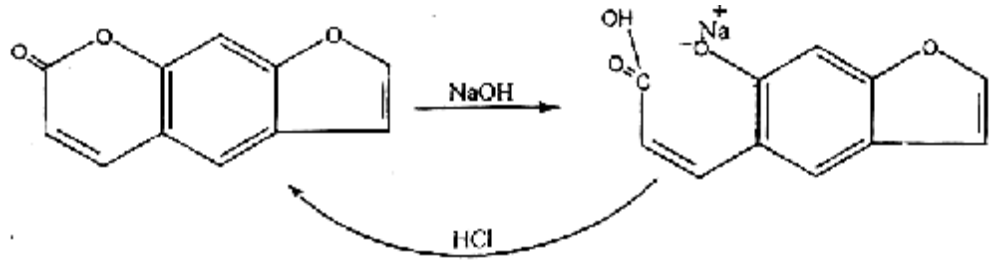
Which are closely related to the psorelan derivatives (furocoumarin)



Ammoidine

9-methoxy – 7H-furo [3,2-g] [1] benzo pyran-7-one

For this reason their behaviors were very close.
Both of them are freely soluble in chloroform. The separation was done by the fact that psoralen derivatives but not the alkaloids, are soluble in aqueous alkaline solution with ring cleavage, and reconstituted upon neutralization [19].



So the mixture was treated with 5% sodium hydroxide solution, and partitioned with equal volume of chloroform. After separating the two layers, the aqueous alkaline layer was neutralized with 5% HCl solution and extracted with chloroform to obtain pore ammoidin with traces of γ-fagarine.
Further purification was done by preparative TLC, and a pure compound was obtained having a sharp melting point of 148°C. Mixed melting point 148°C.
The identity of ammoidin was approved by TLC using two different solvent systems, and was authenticated with a standard reference.
Furthermore, the identity was approved by HPLC the retention time was also authenticated with a standard reference as shown in (Fig-2).
Potentiation of the isolated ammoidin together with the standard compound (Figs-3&4), show a sharp peak with higher intensity.
It is clear that maximum amount was found in the sample collected from Daquk in June. This result is in agreement with a previous work on the alkaloid contents of this plant[24]. It was also found that in June the quantity of alkaloids was in maximum concentration.

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