Phytochemical and Pharmacological Study of Valepotriates in *Valeriana officinalis L.* F.Valerianeceae Cultivated in Iraq.

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Abstract

This study concerned with phytochemical investigation and methods of extraction and separation of active constituents from *Valeriana officinalis* plant cultivated in Iraq. Due to the large number of active constituents in *Valeriana officinalis*, it was necessary to make analytical study of its constituents to determine the chemical nature of these constituents and then determine the main classes (terpenes and iridoids) using chemical reagents specific for each class. Different organic solvents like ethanol (70%) used in soxhlet apparatus and hexane, ethyl acetate and methanol were used separately to extract the main active constituents by maceration. Through comparison between these solvents using thin layer chromatography (TLC), it has been found that hexane was best suited to extract most of the active constituents from the plant by maceration method. Analytical study showed that terpens was separated and purified using preparative TLC and preparative HPLC. Identification of isolated component (valtrate) from roots of plant was obtained using HPLC and PHPLC also depending on some material-specific constants such as infrared spectroscopy (FTIR). Results of analysis showed that *Valeriana officinalis* cultivated in Iraq is a good source of many constituents with different important pharmacological activities. The pharmacological study showed that the total organic plant extract has sleep induction effect when injected I.P. to experimental mice.

Key wards: ***Valeriana officinalis*, Valepotriate, Insomnia**.

دراسة كيميائية فعالة لنبات النارين الطبي المستزرع في العراق

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الخلاصة

تقدم هذه الدراسة بحث شامل للدراسة الكيميائية النباتية وطرائق لاستخلاص و فصل المواد الفعالة لهذا النبات المستزرع في العراق . ونظرا لكثرة المواد الفعالة في نبات النارين الطبي , لذا كان من الضروري اجراء دراسة تحليلية لمكوناته لتحديد الطبيعة الكيميائية لهذه المركبات ومن ثم تحديد أصناف المركبات الفعالة (التربينات والكلايكوسيدات والزيوت الاساسية) باستخدام الكواشف الكيميائية الخاصة بكل صنف.وبعد ذلك تم استخدام مختلف المذيبات العضوية ومنها الكحول الاثيلي (٧٠% ايثانول) للاستخلاص بطريقة (soxhlet) والهكسان و خلات الاثيل والكحول المثيلي لاستخلاص المركبات الفعالة بطريقة النقع البارد ومن خلال المقارنة بين المذيبات بطريقة كروماتوغرافيا الطبقة الرقيقة وجدنا ان استخدام الهكسان هو الانسب لاستخلاص اكبرعدد من المواد الفعالة من النبات بطريقة النقع البارد. اظهرت نتائج فحص المستخلص بطريقة كروماتوغرافيا الطبقة الرقيقة عن وجود مركبات كلايكوسايدية (مجموعة التيربينات) حيث تم فصلها وتنقيتها باستخدام كروماتوغرافيا اعدادي الطبقة الرقيقة مع كروماتوغرافيا اعدادي عالية الاداء السائلة. ثم تم تشخيص المركبات المفصولة من جذور النبات باستخدام طرائق الكروماتوغرافيا الطبقة الرقيقة الحديثة ومنها كروماتوغرافياعالية الاداء السائلة مع كروماتوغرافيا اعدادي عالية الاداء السائلة, ودراسة بعض الثوابت الخاصة بكل مادة مثل مطياف الاشعة تحت الحمراء. وبتحليل نتائج التقييم النوعي التي حصلنا عليها يمكننا القول ان نبات النارين الطبي المستزرع في العراق يعد نموذج غني ومصدر جيد للعديد من المركبات ذات التاثيرات العلاجية المعروفة. وبالنسبة لتاثير النبات وفعاليتة في علاج الأرق والمساعدة على النوم تم اثباته من خلال حقن المستخلص النباتي بطريقة الحقن داخل البيريتون (الصفق) للفيران المختبرية ومراقبة تاثيره عليها.

الكلمات المفتاحية : النارين الطبي ، فاليبوترايت ، علاج العصبية

Introduction

The Plant *Valeriana officinalis* L. from Valerianaceae family, figure (1- A) has been used as a medicinal herb since at least the time of ancient Greece and Rome. Its phytotherapeutical properties were described by Hippocrates as sedative and anti-anxiety [(1)] .The part of the plant used medicinally is the root or rhizome figure (1- B). The rhizome is light grayish brown, about the size of a finger joint, bearing many rootlets. The fresh root has no odor, while the dried root smells distinctly unpleasant, due to isovaleric acid. The plant itself is 50 to 150 cm tall with pinnate leaves and white or pink hermaphroditic flowers with three stamens; the stem is upright and without branches [(2)].

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Valerianaceae a family of 13 genera and about 360 species. Genera include Valeriana (over 200 spp.), valerianella (80 spp.), Centranthus (12 spp.) and Patrina (20 spp.).[3]
The roots of members of the valeriana (valerian) species contain valepotriates, which have alkylting properties. Valtrate/ isovaltrate and dihydrovaltrate are mutagenic in bacterial test systems in the presence of a metabolic activator, and their degradation products baldrinal (from valtrate) and homobaldrinal (from isovaltrate) are mutagenic even without metabolic activation. These latter compounds also have direct genotoxic effects. As far as is known, decomposition of dihydrovaltrate does not yield baldrinals. A freshly prepared tincture contains 11% of the valepotriates originally found in the root material. Storage at room temperature rapidly reduces this to 3.7% after 1 week and 0% after 3 weeks. In view of this rapid degradation, it is not surprising that commercially available tincture samples yield baldrinals. There is substantial variation in the chemical constituents in plants from different sources and growing conditions, processing methods and storage conditions but the differences are small [4] .

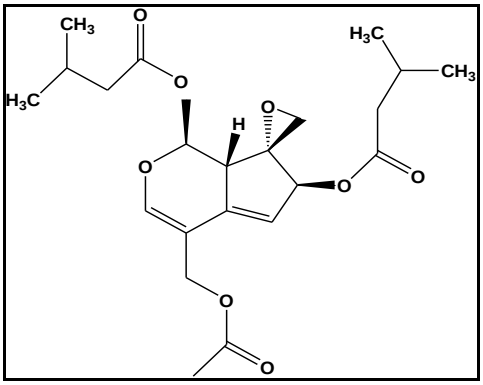
The importance of photoregulation in germination processes was studied by Berbec; He confirmed that, while the light increases the germination percent, it has only a slight effect on the length of period needed for the appearance of the first germ. The effectiveness of light is dependent on temperature conditions. The effectiveness of light proved to be an optimum one at 25 °C, when the germination power (measured on 7th day) was higher by 20 % and the number of seeds which had germinated by the end of experiment was greater by 10—11 percent, compared with dark control [5] .
Valerian root contains bicyclic monoterpenes (valepotriates (0.5% -2.0%) – notably valtrate and dihydrovaltrate), (volatile oils(0.2 – 2.8%)valeranone, valerenal, and valerenic acids), sesquiterpenes, lignans, and(alkaloids(0.05 – 0.1%) actinidine, valerianine and alpha methyl pyrryl ketone).Free amino acids, such as gamma-aminobutyric acid(GABA), tyrosine, arginine, and glutamine are also present [6,7] as shown in fig(2). Epoxy iridoid esters (valepotriates) discovered in 1966, were thought to be the sole active constituents, although their decomposition products, the baldrinal , homobaldrinal and other components are understood to lend therapeutic benefit [8] . valepotriates are common in valerianaceae plant family and considered to be one of the main groups responsible for the sedative activity of valeriana preparations [9] .

Valerian's mechanisms of action are not completely understood. Valerian interacts with neurotransmitters such as GABA [10, 11] and produces a dose – dependent release of GABA [12]. Valerian also inhibits the enzyme-induced breakdown of GABA in the brain, with concomitant sedation [13]. The Greek physician, Dioscorides, apparently recommended valerian root to treat myriad disorders including heart palpitations, digestive problems, and urinary tract infections [14, 15].
In 1998, valerian was the 10th most popular herbal remedy sold in the United States [16].

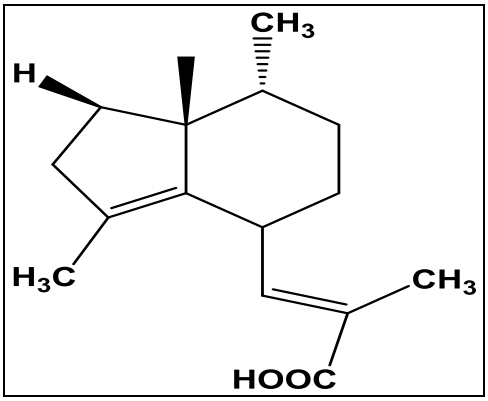
Valerian is frequently listed among the ten most widely used herbal supplements [17]. Historically, valerian has been used as anti-spasmodic, carminative and mild analgesic [18].

Figure (1) A- *Valeriana officinalis* plant

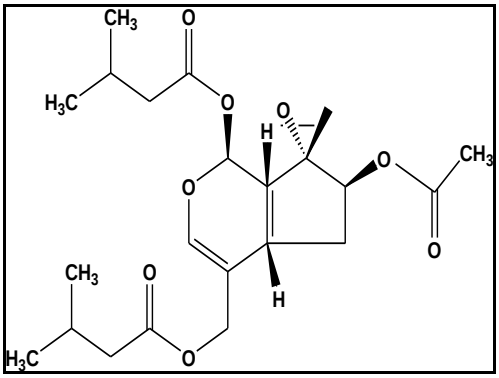
Figure (1) B-dried root of *Valeriana officinalis*



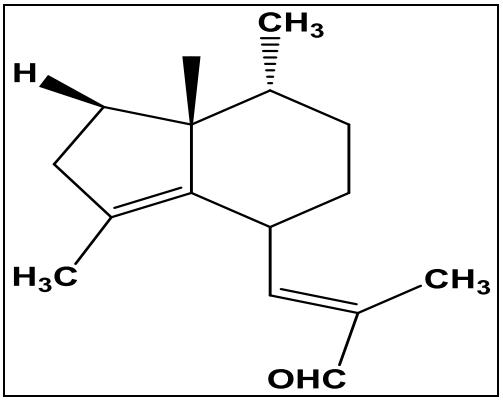
Valtrate



Valerenic acid



Didrovaltrate



valerenal

Figure (2) active constituents of *Valeriana officinalis.*

Experimental

Plant materials

The cultivated whole plant was collected from the department of the medicinal plants, College of Agriculture, University of Baghdad. The plant material (root) was collected during May (2013) and dried at room temperature in the shade, then pulverized by mechanical mills and weighed

Instruments

Electrical sensitive balance: Sartorius/ Germany, Ultraviolet light: Desaga Heidelberg/ Germany, Magnetic stirrer: Heidolpha / Germany, Rotatory evaporator: Buchi Rotatory evaporator, Chiller: Ultratemp 2000, Water bath: Memmert/Germany, Sonicater –ultrasonic cleaner: sonication of extract was carried out using (Copley scientific, UK.), FTIR: Shimadzo FT-IR-8400s IR Spectrometer, HPTLC: Eike Reich/CAMAG – Laboratory, PHPLC: PHPLC analysis was carried out by (JASCO-FC2088-30)/Japan,HPLC: Shimadzo L20204806962: HPLC analysis was done using Column: Apex (Jones Chromatography, Hengoed, UK) [(19)].

Experimental animals

Healthy adult albino male mice were obtained from The Animal House of College of Pharmacy, University of Baghdad. The weight of mice were 25±5 gram, the animals were housed in groups of eleven per cage, with light/dark period of 12 hours. They were provided with food and water ad libitum. All experiments were conducted between 8:00 am and 2:00 pm. All animals were carefully monitored and maintained in accordance with the ethical recommendation of College of Pharmacy.

Chemicals

All chemicals used were analytical grade, supplied from warehouse chemicals of College of Pharmacy/ University of Baghdad and valtrate (98%) standard from Jinan Boss Chemical Industry/ China.

Extraction of plant

Method NO.1

The dried and powdered roots of *Valeriana officinalis* (25g) were extracted in a Soxhlet extractor with 70% ethanol (105 ml) (70-80 C$^{\circ}$) for 4 h. The organic extract was evaporated to dryness by using rotary evaporator. The dried and powdered roots (15g) were boiled in water (250 ml) for 30 min. The decoction was evaporated to dryness in water bath. The dried samples were kept in sealed bottles under vacuum to prevent evaporation of solvents and plant fermentation. The residues were suspended

in distilled water (3 ml for aqueous extracts and 5 ml for ethanol extracts). The two valerian extracts of ethanol and aqueous residue were extracted at room temperature with dichloromethane (3 x 25 ml, 20 min each) in an ultrasonic bath and isolated by separatory funnel. The combined extracts were filtered and evaporated to dryness and kept for chromatographic analysis [20].

Method NO.2

Twenty gram of dried powdered of root of *Valeriana officinalis* was extracted with n-Hexane (30 ml) with occasional stirring overnight by using magnetic stirrer then filtered and keep in sealed bottle in refrigerator for analysis. Then the drug was extracted with ethyl acetate (30 ml) with occasional stirring overnight, filtered and keep sealed in refrigerator for analysis and finally drug was extracted with methanol (30ml) with occasional stirring overnight, filtered and kept sealed in refrigerator for chromatographic analysis [21].

General phytochemical screening by chemical tests

The root part of plant has been screened for alkaloids, terpens, iridoids, saponines, tannins and essential oils.

A- Vanillin $-H_2SO_4$ reagent for Terpens. [22].
B- 2, 4 Dinitrophenyl hydrazine for Valepotriates.
C- HCL-acetic acid reagent & anisldehyde reagent for valerenic acid.
D- Reagent for alkaloids [23].
E- Ferric chloride test for tannins.
F- Froth test for saponin.

Chemical Test (Detection of valepotriates)

About 5 ml of dichloromethane added to 0.2 g of freshly powdered root, let stand for 5 minutes, shake several times, and filtered. Rinse the marc with 2 mL of methylene chloride and added the rinse to the filtrate. Collected the filtrate and washings in a test tube and blow dry to remove the solvent. Dissolved the residue in 0.2 mL of methanol. Added 3 mL of a mixture of equal volumes of chilled acetic acid and hydrochloric acid to 0.1 mL of the methanol. Shake well. If valepotriates are present, the solution will turned in a blue color within 15 minutes [24].

Isolation and identification of active constituents

Separation of the main active constituents of *Valeriana officinalis* L. root was carried out using Preparative TLC**:** TLC plates, 20x20cm and 1mm thickness of silica gel GF254 developed in solvent system (hexane : ethyl acetate : acetic acid 65:35:0.5) with standard reference. The chromatogram was visualized by UV lamp (at 254 nm and 366 nm) [25]. Identification of active constituents was done by:

1-Matching with standard by TLC using the following mobile phases:

S_1 Hexane: ethyl acetate: glacial acetic acid (65:35:0.5), S_2= Hexane: ethyl methyl ketone (80:20).

2-High Performance Liquid Chromatography (HPLC): HPLC analysis was done using Column: Apex (Jones Chromatography, Hengoed, UK) ODS (C18, 5 Mm, 4.6 mm i.d X 250 mm) pump: Shimadzu (Columbia, MD, US). LC-10AT. Detector: Shimadzu SPD-10A, (221) nm. The injected volume was 20 Ml. The eluent was methanol/water (0.5% H_3PO_4, pH 2) 80: 20. The flow rate was 1.5 ml/min [19].

mobile phase was methanol/water (0.5% H3PO4, pH 2) 80: 20.

3- Preparative High Performance Liquid Chromatography (PHPLC): PHPLC analysis was carried out by (JASCO-FC2088-30)/Japan, injected volume was 2 ml. The eluent was methanol/water (0.5% H_3PO_4, pH 2) 80: 20The flow rate was 10 ml/min .

4- Fourier transforms infrared spectroscopy (FTIR) in KBr disk.

5-High performance thin layer chromatography (HPTLC): the mobile phase is hexane: ethyl acetate: acetic acid (65:35:0.5), for valtrate standard, hexane & organic extract sample [24].

Pharmacological study

Experimental test (Sodium phenobarbital-induced sleeping time test):

Mice were divided into three groups (11 animals each). Groups were received either 200 mg/kg I.P. Extract (Extract-treated group), or 10 mg/kg I.P. diazepam (Diazepam treated group). In addition to the control group which received vehicle (distilled water) I.P. (control group) One hour later, all groups received 50mg/kg I.P. phenobarbital, and sleep were recorded. The time elapsed between the loss of and recovery from the righting reflex was recorded for control and treated animals [26].

Results

The preliminary phytochemical investigation of valeriana root revealed the presence of terpens (valepotriates), iridoids, and essential oil as main constituents while alkaloids present in a very low percentage, tannins and saponine absence, show in table (1).

Table (1): The results of the general screening by chemical tests.

Plant	Plant part	Alkaloids	Terpenes	iridoids	tannins	saponines
Valeriana officinalis	Root and rhizomes	+	+	+	-	-

Identification of isolated compound (valtrate) depends on

1-Qualitative analysis under UV light (254 nm) revealed the largest zone at Rf 0.6 due to valtrate. As shown in Fig (3).

2- Qualitative analysis under UV light (254 nm) revealed the R_f value at 0.6 due to valtrate from organic (ethanol) extract with reference standard as shown in Fig(4).

3- Qualitative analysis can be done by HPLC (High performance liquid chromatography) analysis by comparison of retention time of analyzed sample and valtrate standard at identical chromatographic conditions. The results are shown in Figure (5) and table (2).

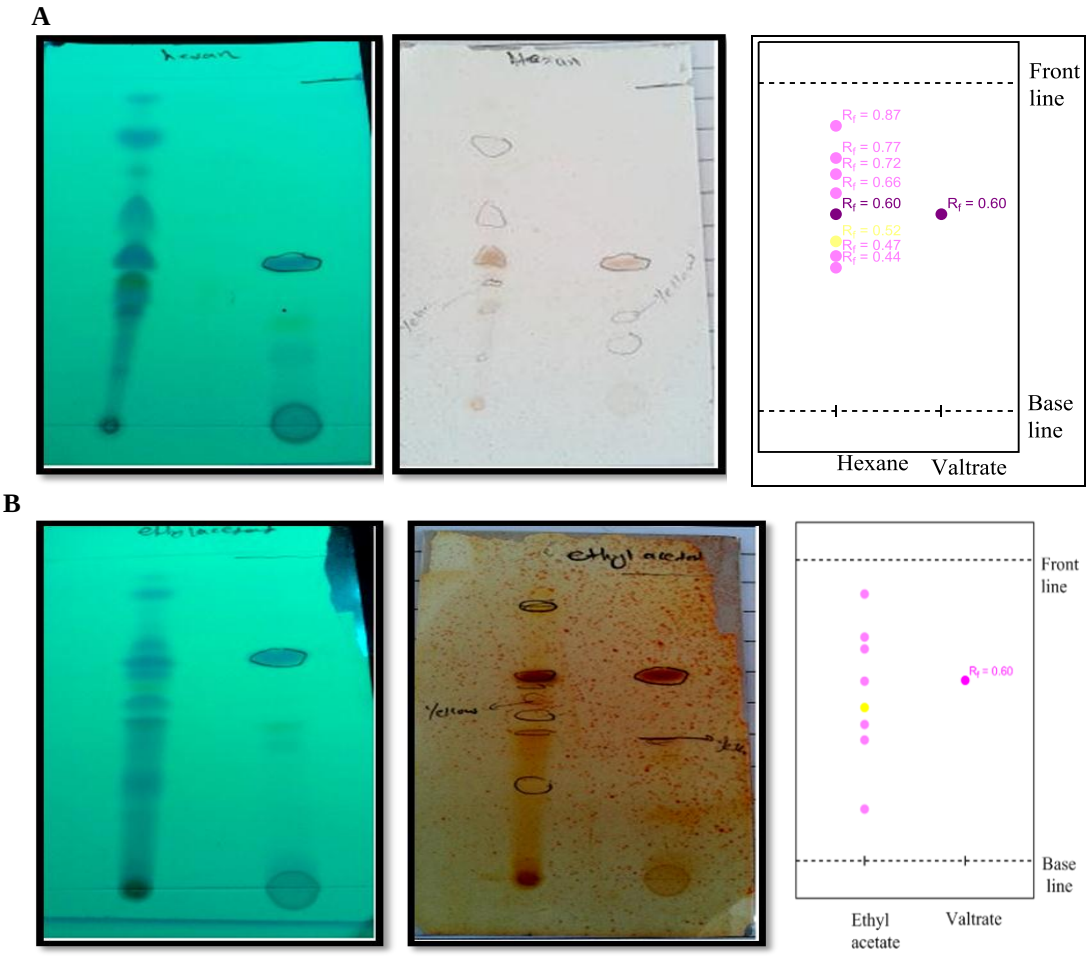


Figure (3) thin layer chromatography for (A) hexane extract and after spraying by anisldehyde H2SO4 reagent. (B) Ethyl acetate extract and after spraying by anisldehyde H_2SO_4 reagent. With valtrate standard on silica gel GF_{254} developing in S_1 solvent system and detection by UV light at 254nm.

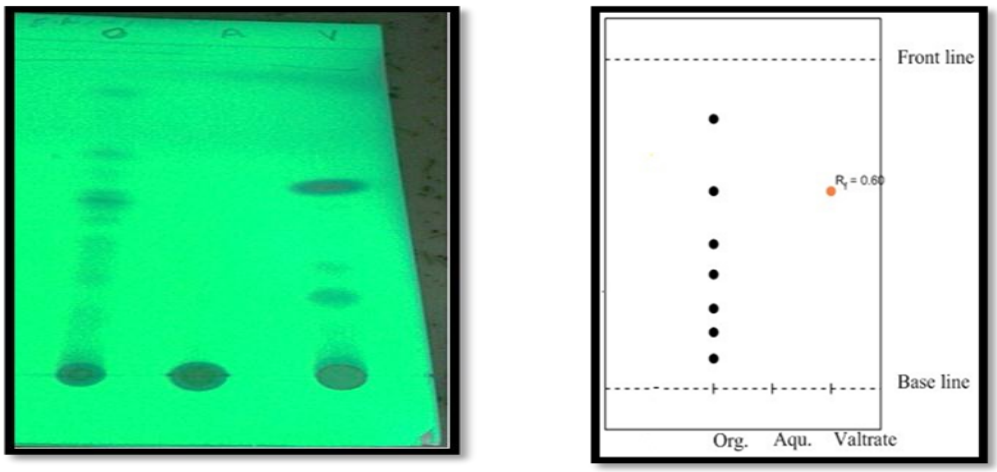
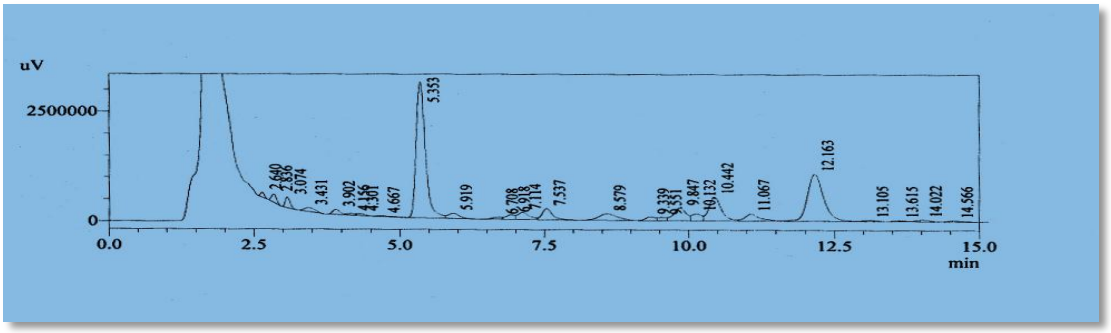


Figure (4) thin layer chromatography for aqueous and organic extracts with valtrate standard on silica gel GF_{254} developing in S_1 solvent system and detection by UV light at 254nm.

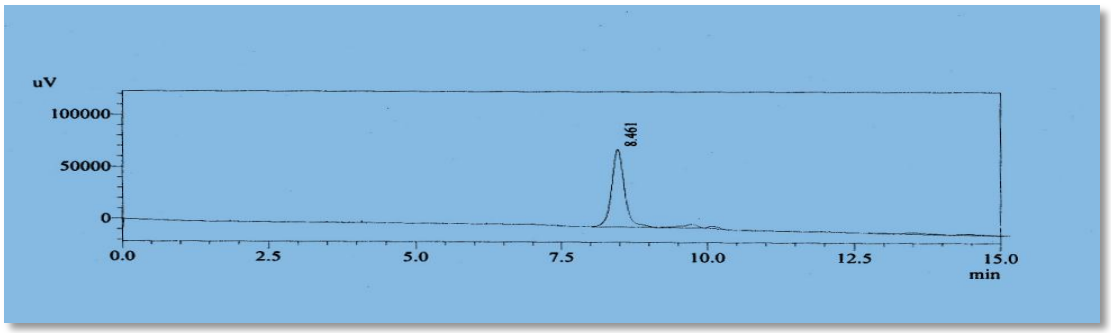
Table 2: The retention time of isolated compound compared with that of reference standard.

Compound	R_t of root extract	R_t of valtrate standard
Valtrate	8.461	8.579

A



B



C

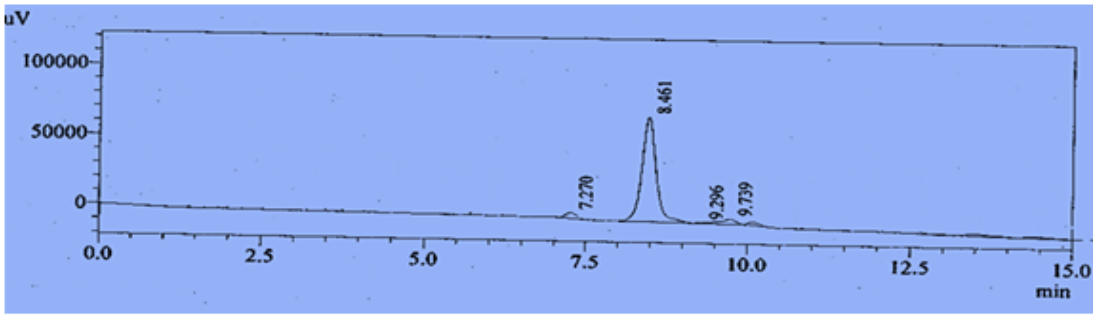


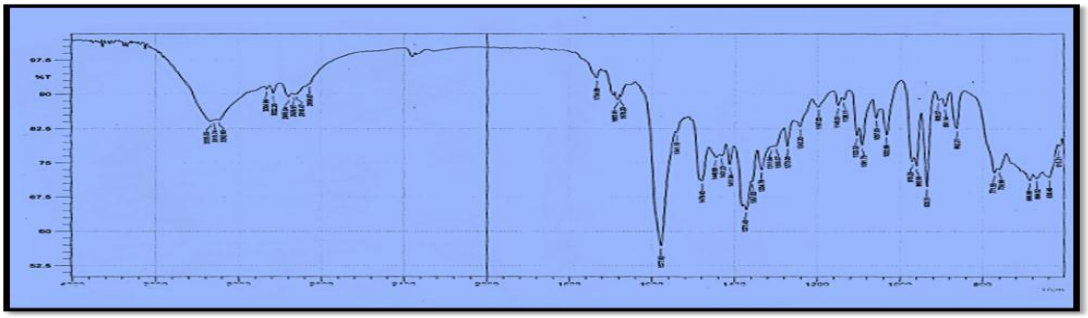
Figure (5) HPLC analysis of (A) organic extract (B) valtrate standard and (C) isolated valtrate

4- FT-IR spectroscopy: Many functional groups can be identified by their characteristic vibration frequencies. As shown in table (3) and fig (6).

Table (3): IR absorption bands of isolated compound (in cm^{-1}).

Compound	Approximate positions of characteristic bands.
valtrate	1734.06(C=O), 1573.97-1543.10 (Ar.C=Cstretching),1377-1365(isopropyl), , 2916 , 2850 (C-H stretching /-CH3), ,1438 , 1365 (C-H bending /-CH3) , 2955 (C-H stretching /-CH2)1469.81 (C-H bending /-CH2)

A



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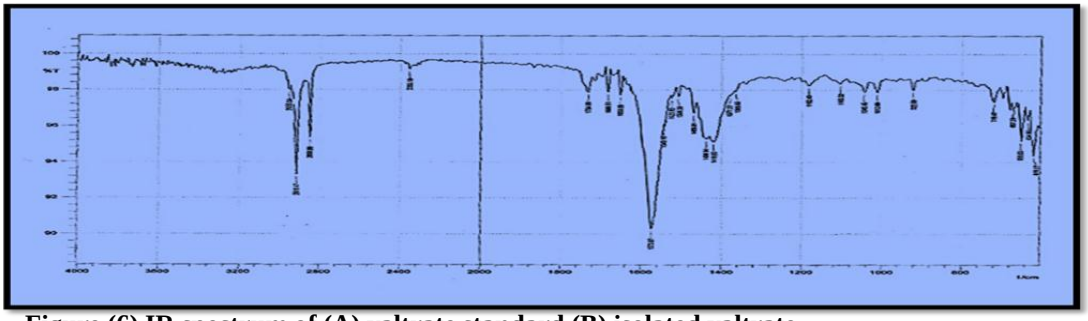


Figure (6) IR spectrum of (A) valtrate standard (B) isolated valtrate

5- HPTLC was carried out for further identification of main active constituents present in hexane and ethanol extracts of *Valeriana officinalis* roots with (valtrate) in S_1 solvent system. As shown in Fig (7).

A B

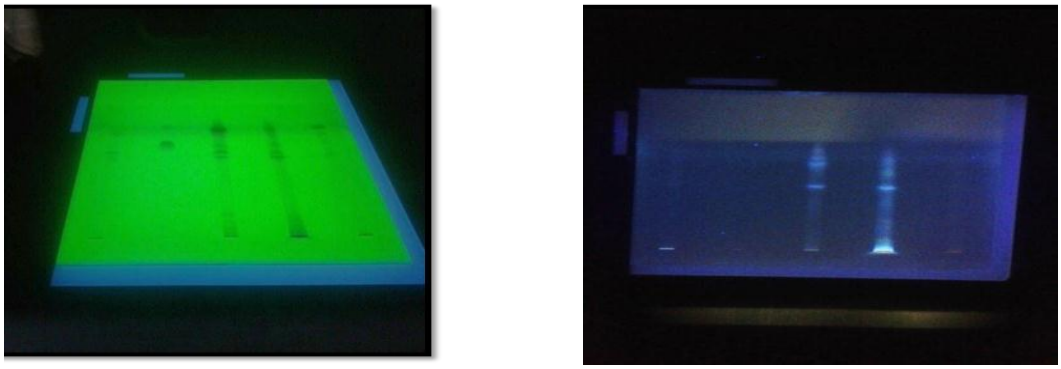


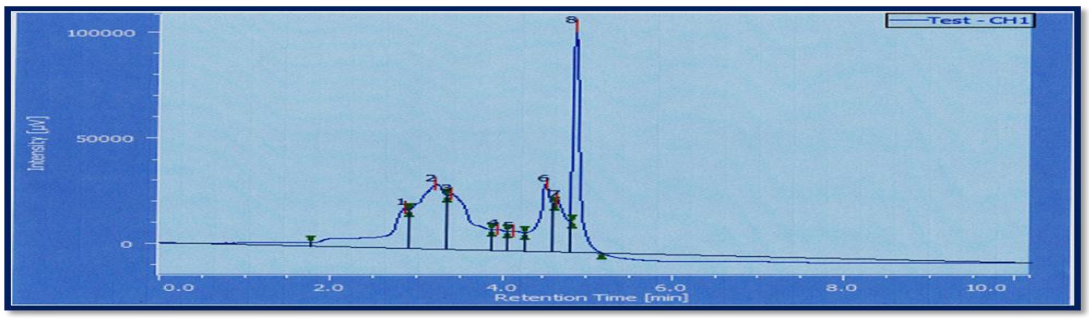
Figure (7) HPTLC plates of plant extract with valtrtae standard developing in S_1. Detection under UV light at (A) 254 nm and (B) 366 nm.

6- Preparative HPLC typically involves working with samples at their maximal concentrations and column loading far above the linear adsorption isotherms required for analytical purposes. As shown in fig (8) and tab (4).

Table (4) Retention time of organic root extract and isolated compound (valtrate)

compound	R_t of root extract	R_t of valtrate standard
valtrate	3.933	3.892

A



B

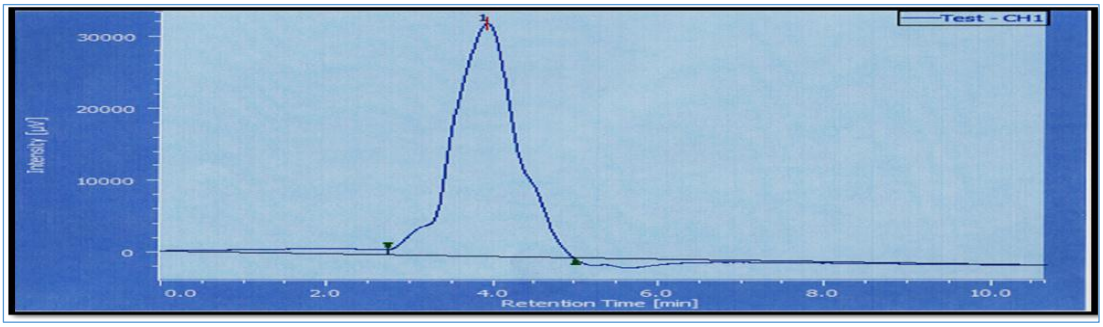


Figure (8) PHPLC for (A) organic extract and (B) valtrate standard

Sodium Phenobarbital-Induced Sleeping Time Test:

In this study one-way ANOVA showed significant differences in the sleeping time between the treated groups. Tuckey's *post hoc* test revealed that there was significant differences in the sleeping time between extract treated group compared to control group. Furthermore, diazepam treated group was significantly different from control group. There was no significant differences between extract and diazepam treated groups.

The results of the present study showed that animals of extract treated group slept faster than animals of other groups. Righting reflex test is a simple, rapid test to assess loco motor abilities in mice. It evaluates general body strength by scoring or measuring the ability of mice to return to their four paws after having been placed in a supine position or on their side [27]. These results suggest that the cultivated *Valeriana officinalis* in Iraq that been used in this study has hypnotic effect at 200 mg/kg dose of extract. As in figure (9).

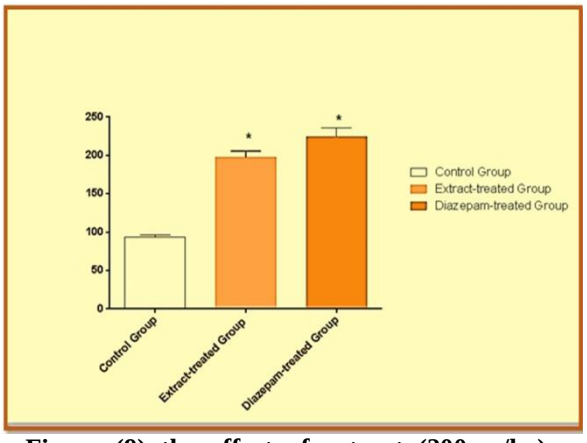


Figure (9) the effect of extract (200mg/kg) and diazepam (10mg/kg) on phenobarbital induced sleeping time in mice. Extract as well as diazepam were significantly differ from control group *p< 0.05.values are expressed as mean+ SEM (n=11).

Discussion

Phytochemical analysis showed that Ethanol 70% was used for extraction of the main active constituents (valepotriates) by soxhlet apparatus from the dried plant material in extraction method (No.1). Different solvents were used with increasing polarity (hexane, ethyl acetate and methanol) in method (No.2) to

get different fractions with different active constituents. From Previous analytical data hexane and ethyl acetate extracts separated and isolated more components than organic extract in S_1 solvent system due to like dissolve like rule in which S_1 contained hexane and ethyl acetate portions so more components separated in TLC in addition to the nature of *Valeriana officinalis* components in which there is a balance in lipophilic and hydrophilic groups in most of its chemical structure so extraction with hexane and ethyl acetate gave more separated components because there was different solvents (hexane-non polar and ethyl acetate –polar) while extraction with ethanol by soxhlet apparatus ,only one polar solvent(ethanol) used so less components separated in TLC .The compounds present in the root of valeriana are involved in its pharmacological action, especially Valerenic acids and Valepotriates, which promote the inhibition of degradation of gamma –amino butyric acid [(28)]. The physiological mechanisms of the action of valeriana on CNS involved in the action of GABA potentiating or a direct action at the site of receptor [(29)]. Although valepotriates were once thought to be the active ingredients, these compounds are chemically unstable: Instead, their degradation products, baldrinals, are found in number of preparations, and may account for much of valerian's sedative effect [(30)].

Conclusion

Our results demonstrated that the extracts of Iraqi *V. officinalis* roots possess significant properties due to the structural features of the active principles they contain. Extracts enhance or potentiate sleep tendency to mice, but the potency differed considerably. The different constituents of organic, aqueous, hexane and ethyl acetate extracts may be related to differences in the extraction procedure and therefore in their qualitative chemical profiles but all of these extracts indicated presence of valtrate in those extracts samples.

Acknowledgements

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