

Original Research Article

Detection and Estimation of Vitamin D2 in *Catharanthus Roseus* by HPLC and other Molecular Spectra Instruments as A Source of Vitamin D3 Production

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Abstract

Vitamin D is one of fat-soluble vitamins, considered a steroid hormone. It is regulate and responsible for improving the metabolism and absorption of calcium, iron, magnesium, zinc, and phosphate in small intestine. SHIMADZU High-Performance Liquid Chromatography was used for qualitative and quantitative estimation of vitamin D2 in aqueous extracted of *Catharanthus Roseus* plant; (20µl, 2.5 IU/gm) standard material of pure vitamin D2 (Ergocalciferol) injected and measured at reference conditions to fix retention time (RT), then 20µl of extracted plant sample was injected and measured at the same conditions.

An intensive analysis conducted on aqueous, alcoholic extract and dry powder of plant leaves using modern and sophisticated instruments. Quantitative results from HPLC shows that Vit.D2 in drying weight of plant up to 187.840 IU/gm. Qualitative results from HPLC, UV-VIS, IR, ATR-FTIR shows match the effective groups of pure standard Vit.D2 and alcoholic extract, dry powder of *C. Roseus* plant.

This study aimed to investigate the presence of vitamin D2 in *C. Roseus*, in addition to qualitative and quantitative detection in aqueous, alcoholic extract, and dry powder of plant leaves to use it in practical as a major source for Vit. D3 production in industrial scale; in order to use it in the medical, pharmaceutical and what is called alternative medicine (Herbs) fields.

Key words: *Catharanthus Roseus*, Vitamin D2, Ergocalciferol, Cholecalciferol, HPLC, Molecular Spectra instrument.

الخلاصة

فيتامين D هو من احد الفيتامينات الذائبة في الدهون، ويعتبر من ضمن الهرمونات الستيرويدية. ان فيتامين D هو المسؤول عن تنظيم وتحسين التمثيل الغذائي وامتصاص الكالسيوم والحديد والمغنيسيوم والزنك والفوسفات في الأمعاء الدقيقة.

في هذه الدراسة تم استخدام جهاز كروماتوغرافيا السائل العالي الاداء نوع SHIMADZU للتقدير الكمي والنوعي لفيتامين D2 في نموذج المستخلص المائي لنباتات عين البزون. بحيث تم حقن (20µl ، 2.5 وحدة دولية / جم) من المادة القياسية لفيتامين D2 النقي (إرغوكالسيفيرول) وتمت عملية القياس وفقا للشروط المرجعية والظروف العلمية الخاصة بهذا الفيتامين، لتحديد وثبتت زمن الاحتجاز الحقيقي (RT). بعد ذلك تم حقن 20µl من نموذج المستخلص المائي للنبات وتمت عملية القياس تحت نفس الظروف.

أجريت دراسة مكثفة على المستخلص المائي والكحولي والمسحوق الجاف لأوراق النبات باستخدام أجهزة حديثة ومتطورة. ان النتائج الكمية التي تم الحصول عليها من جهاز HPLC تبين أن نبات عين البزون يحتوي على تركيز فيتامين D2 يصل إلى 187.840 وحدة دولية / جم من وزن النبات الجاف. ان التحاليل والفحوصات النوعية المستحصلة من الاجهزة السابقة تظهر تطابق المجاميع الفعالة في المستخلص الكحولي والمسحوق الجاف لنبات عين البزون مع المادة القياسية لفيتامين D2.

هدفت هذه الدراسة إلى التحقق من وجود فيتامين D2 في نبات عين البزون، بالإضافة إلى الكشف النوعي والكمي في المستخلص المائي، الكحولي، إضافة إلى المسحوق الجاف من أوراق النبات باستخدام الأجهزة المبينة أعلاه للتأكد من مدى إمكانية استخدامها عملياً باعتبارها مصدراً رئيسياً لفيتامين وإنتاج D3 في نطاق صناعي. من أجل استخدامها في مجال الطب والأدوية وما يسمى بالطب البديل (طب الأعشاب).

الكلمات المفتاحية: نبات عين البزون, D, اركوكالسيفيرونول, كوليكالسيفيرونول, جهاز السائل العالي الاداء, اجهزة الاطراف الجزئية.

Introduction

There were a lot of plants are considered as one of the most important sources to produce drugs and medicines manufacture, because of highly contains chemical values, biological and effective compounds in addition to the construction of these compounds have been naturally and their concentrations are somewhat low, which reduces the adverse side effects caused by the chemically manufactured medicines, so it has adopted in the preparation of many of medicines and medical drugs. At recent years; Scientists, researchers, and the biggest pharmaceutical companies interested toward medicinal plants after proving their activity in preparation of a lot of drugs and medical compounds in addition of speed therapeutic effect and the lack of negative side effects, so there became it's own medicine is called alternative medicine and became its own medications called Herbal Medical Therapeutics, thus become one of the remedies for many disease [1].

Catharanthus Roseus- scientific name- is considering one of the most important medicinal plant in the world. Belong to Apocyanaceae Family, which includes five types. Also known (Periwinkle Rose, Vinca Rosa) [2]. Flowering circular shape composed of five petals with several colors, including white, red, pink and purple, native to Madagascar's original south-east and east, the plant is grown easily [3-4]. Binomial Name- *Catharanthus roseus* (L.) G. Don. *C. roseus* is an evergreen sub herb or

herbaceous plant growing to 1 m. tall, the leaves are oval to oblong, 2.5- 9.0 cm. long and 1- 3.5 cm. broad glossy green hairless with a pale midrib and a short petiole about 1- 1.8 cm. long and they are arranged in the opposite pairs[2]. Classified as in following: Domain: Eukarya: eukaryotes Kingdom: Plantae: plants. Subkingdom: Tracheobionta: vascular plants. Superdivision: Spermatophyta: seed plants. Division: Magnoliophyta: flowering plants. Class: Magnoliopsida: dicotyledons. Subclass: Asteridae. Superorder: Gentiananae. Order: Gentianales. Family: Apocynaceae: dogbane. Subfamily: Rauvolfioideae. Tribe: Vinceae. Genus: *Catharanthus* G. Don. Specific epithet: *roseus* (Linnaeus) G. Don. Botanical name: *Catharanthus roseus* (Linnaeus) G. Don (1837) [5]. The root of *C. roseus* is toxic, bitter, acidic, stomachic and used as a tonic. In Hawaii, the plant is boiled to make a poultice to stop bleeding, in China, it is used as a homemade cold remedy to ease lung congestion, inflammation and sore throats and in the Caribbean, an extract from the flowers was used to make solution to treat eye irritation and infection [6].

Now it is grown in almost worldwide [7], *C. Roseus* has been used in Madagascar, also in many countries in recent time, a popular diabetes therapy, blood pressure, asthma, constipation, liver disease, kidney disease, cancer, and to treat problems of menstruation cycle due to medicinal contains[2].



Figure1: *Catharanthus Roseus*

C. Roseus is containing many important compounds such as Indolealkaloids, carbohydrates, saponin, Tannin, phenols and flavonoids, which are plants' derivatives from multiple hydroxyl and glycosides flavonoid derivatives. Alkaloids are most effective chemical components of this plant, more than 200 Alkaloids are present in, which are used in the pharmaceutical, flavors, smells, food additives, in addition to pesticides and agricultural chemicals[2]. Vitamin D is one of fat-soluble vitamins, considered as steroid hormone. It is regulate and responsible for improving the metabolism and absorption of calcium, iron, magnesium, zinc, and phosphate in small intestine[8]. Vitamin D is an organic chemical compound (or group of related compounds). The most important compounds of this group are vitamin D3 (Cholecalciferol) and vitamin D2 (Ergocalciferol). Both of these vitamins can be obtained from food and dietary supplements [9,10]. It creates (specifically cholecalciferol) in the skin, it is the main natural source of this vitamin, which creates from cholesterol. This process depends on exposure to sunlight (UV specifically), so vitamin D called sun-ray vitamin. Vitamin D which comes from diet or through synthesis in the skin by effect of sun's ray is not biologically active. The activating process of this vitamin require enzymatic conversion processes (hydroxylation) which is made by two steps, first in the liver and the second in the kidneys [11].

Vitamin D is considered as a hormone because it is synthesized in a place and organizes, effects on organs, glands, and places in various parts of the body [12]. It is unique because it can be taken as cholecalciferol vitamin D3 or Ergocalciferol vitamin D2. In addition, like other vitamins, in the modern world are adding vitamin D to the basic foodstuffs, such as milk, to avoid diseases caused by deficiency [9].

Types and forms of vitamin D:

There are multiple forms of vitamin D, starting from 1-5 and most important two forms which they are vitamin D2 or Ergocalciferol (molecular formula: $C_{28}H_{44}O$, molecular weight of 396.648g/mol), and Vitamin D3 or cholecalciferol (molecular formula: $C_{27}H_{44}O$, molecular weight of 384.637g/mol). Vitamin D without subsequent digital indicates to either D2 or D3 or both, known collectively as calciferol [10]. Chemically, vitamin D2 has been identified in 1932, while chemical structure of vitamin D3 was confirmed at 1936, and proved to be a result of ultraviolet radiation of 7-dehydrocholesterol. Chemically, different forms of vitamin D are secosteroid; steroids, such as steroid ring broken links [11]. The difference between structural composition of vitamin D2 and D3 in side of two series. Side chain of vitamin D2 contains a double bond between carbon atoms 22 and 23, and methyl group at carbon 24, as shown in figure 2.

Sources of vitamin D

Food sources of Vitamin D, include: Eggs, Fish, Cod liver oil, Fortified milk and supported breakfast cereals. Daily exposure to the sun for 10 minutes contribute to stimulating the third type of vitamin D production (cholecalciferol) in the skin under the radiation effect of UV on cholesterol, but the second mode of vitamin D (ergocalciferol) are produced in some algae and plants such as ergot plant [10,14].

Biosynthesis and the production of vitamin D

The synthesis and the production of vitamin D3 cholecalciferol by the ultraviolet rays of predecessor, 7-dehydrocholesterol, this molecule occurs naturally in animal's skin and milk. Vitamin D3 can be configured via the skin is exposed to ultraviolet rays, or by exposing the milk directly to ultraviolet light, which is considered one of the trade routes [7,8,10].

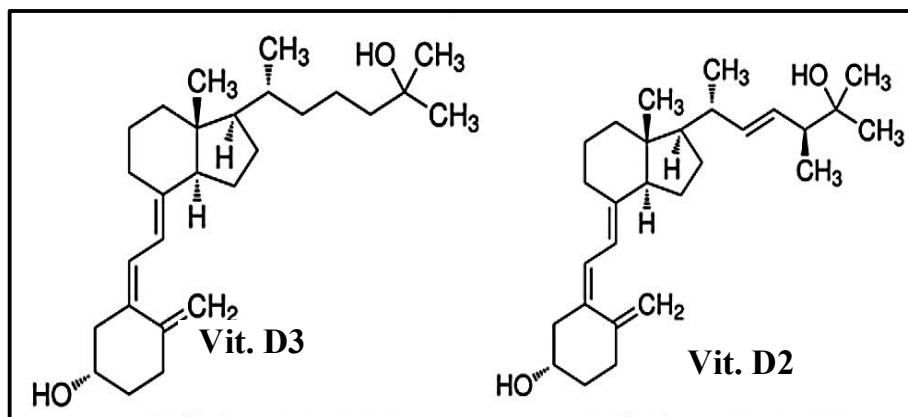


Figure 2: Chemical formula of Vit.D3, D2 from Chen et al. 2010 [15]

Vitamin D2 is derivative of ergosterol, a membrane sterols of the ergot fungus which is produced by some organisms, such as phytoplankton, invertebrates, and yeasts, such as high-end mushrooms and fungi. The production of vitamin D2 ergocalciferol in all of these objects from ergosterol, in response to ultraviolet light. However, like all forms of vitamin D it cannot be manufactured without the UV rays, nor is the production of vitamin D2 of the wild plants or vertebrates because they lack of ergosterole precursors [16].

Industrial production

The production of vitamin D3 (cholecalciferol) industrially through exposure of 7-dehydrocholesterol to ultraviolet light, followed by sterilization. The compound 7-dehydrocholesterol is a natural substance found in the wool fat (lanolin) in sheep wool and other animals. The production of vitamin D2 (ergocalciferol) in the same way using ergo

sterol of yeast or fungus as the raw material [17].

Materials and Methods

Plant: fresh samples of *C.roseus* were collected from local markets at Baghdad city, then washed carefully by tap water to get rid of impurities and dust, later washed again with distilled water, washed and cleaned samples dried and stored at (-20°C) in Polyvinyl bags until time of analysis.

Chemicals: in the present study, the chemicals that used were obtained from thoughtful international companies were high purified as follows; Acetonitrile (C_2H_3N), Methanol (CH_3OH), and Ethanol (C_2H_6O) for HPLC. In this study another chemicals were used as following: standard Vitamin D2 (Ergocalciferol $C_{28}H_{44}O$), Tartaric Acid ($C_4H_6O_6$), Ethyl Acetate ($C_4H_8O_2$), and Ortho-Phosphoric acid (H_3PO_4).

Instrument:- Instrument that have been used in the present study were modern and sophisticated analysis instruments as follows; Preparative and Revers Phase-High Performance Liquid Chromatography (Pre-HPLC, RP-HPLC), UV-VIS Spectrophotometer, IR-Prestige, FTIR BRUKER tensor 27, Freeze Dryer ALPHA 1-4 LD plus, Lovibond pH meter 200, Magnetic Stirrer with Hot plate, Sensitive Balance, Centrifuge Hettich EBA 20, and Water Bath Grant.

HPLC Technique

Detection and Estimation of Vitamin D2 in *C. roseus* plant using Reversed-Phase High-Performance

Liquid Chromatography(HPLC) system in which standard material and Samples were analyzed. SHIMADZU model 10AV-LC equipped with binary delivery pump model LC-10AV, the eluted peaks were monitored by UV-VIS detector SPD-20A. Under the following conditions of separation; Mobile phase was prepared from a mixture of Acetonitrile: Methanol (75: 25 ml), Column Type that used in this study was C18 – ODS (25cm x 4.6 mm X

5µm) with 20 µl as injection volume of sample with Flow Rate 1 ml/min at 30°C through UV detector Spectrophotometer at wave length 265 nm [18].

Preparation and analysis of standard material

Preparation of 2.5 IU/gm, an original standard powder (Ergocalciferol) 100,000 IU/gm from Lyphar Biotechnology in China was used, the standard was dissolved in ethanol, and filtered by (0.45µm) What man filter paper, then passed through Millipore Syringe filter 0.22 µm for HPLC analysis. (20µl, 2.5 IU/gm) standard material of pure vitamin D2 (Ergocalciferol) injected and measured at reference conditions to set and fix the real retention time (RT), then 20µl of extracted plant sample was injected and measured at the same conditions.

Separation Conditions were; Acetonitrile: Methanol (75: 25 ml) as isocratic mobile phase, column was C18-ODS (25cm x 4.6 mm x 5µm) with 20 µl as injection volume with 1ml/min flow rate at 30°C through 265 nm wave length UV detector Spectrophotometer [18].

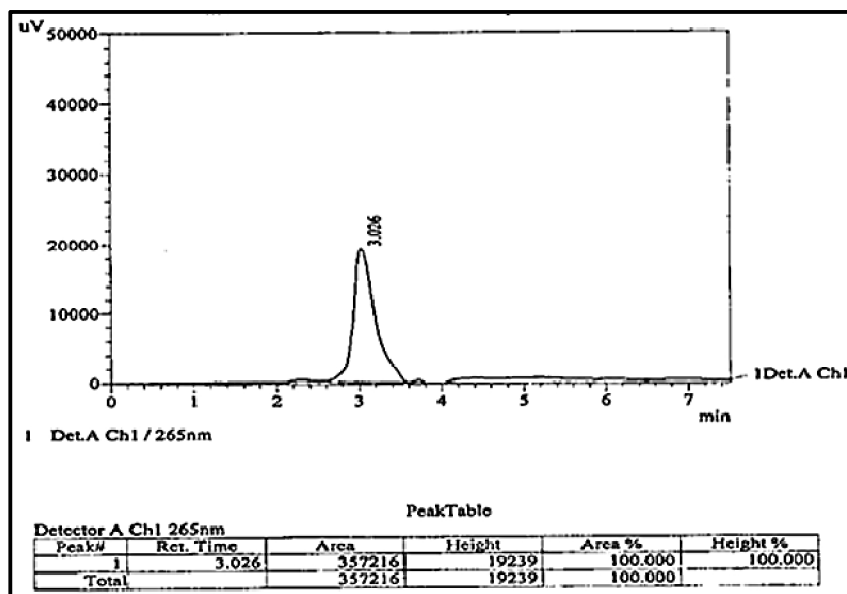


Figure 3: represent peak top of standard vit.D2 in RP-HPLC

Aqueous extraction, detection and estimation of vitamin D2 in the *C. roseus* plant using (RP-HPLC) [19].

From frozen samples of *C. roseus* in (-20), a weight of 50 g from stems, leaves, and flowers, then placed directly in electric blender, then a volume of 500

ml distilled water was added and mixed for 5 minutes, sample placed on magnetic stirrer for 72 hours at 37°C. Sample must be well covered and protect from oxidation by oxygen, and prevent conversion by ultraviolet radiation of sun light. Sample was filtered by Buchner funnel and passing into activated charcoal, then filtered by What man filter Paper 0.45µm and Millipore filter (0.22 µm), volume of 20µl from aqueous extract sample was

injected into(RP-HPLC) under the same conditions of standard analysis.

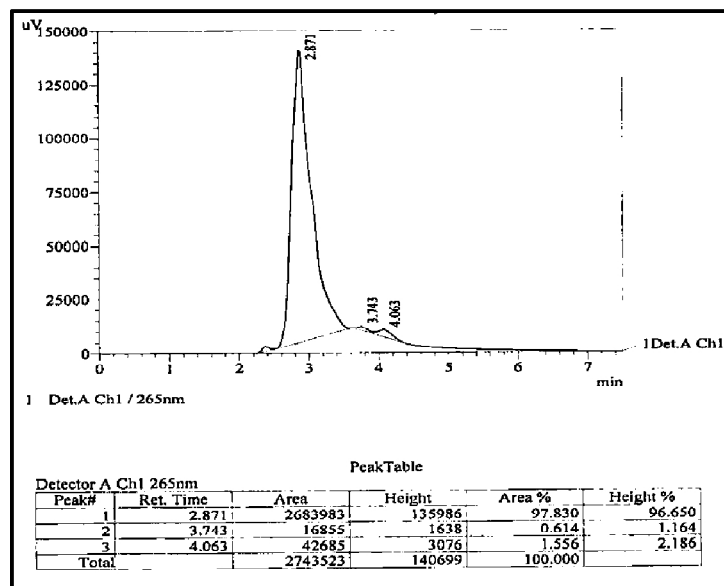


Figure 4: represent peak top of *C. roseus* aqueous extract sample in RP-HPLC

Detection and Estimation of Vitamin D2 in *C. roseus* Using (UV-VIS) [20,21]

UV-Vis absorption spectra were collected at 30°C using PHILIPS UV-VIS double beam spectrophotometer equipped with a single position. Quartz cuvette with a 1 cm path length was used for all UV-VIS experiments.

Sample preparation and extraction

C. roseus samples dried in shade at room temperature for a week within well-ventilated environment, with turning plant

parts to avoid fungal growth, dry parts of the plant (stem, leaves, and flowers) grind with ceramic mortar. A weight of (25 gm) of grinded dry plant was placed into thumble. A volume of (250 ml) from ethanol was added into round bottom flask to use it in Soxholet escalation for (12 hours) at 80°C., all extract was evaporated by rotary evaporator and the sample was transferred to freeze dryer to conversion it into powder for using in other experimental tests.

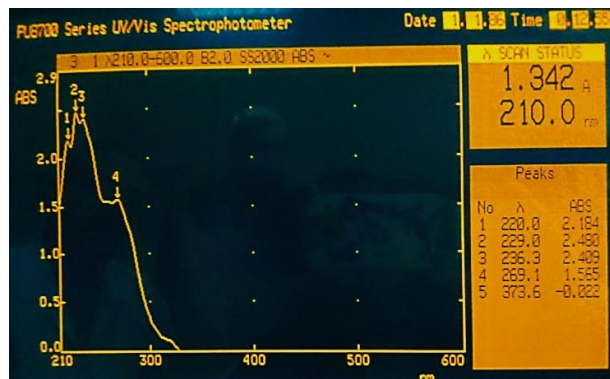


Figure 5: Absorbance peaks level for Standard vit.D2 by UV-VIS

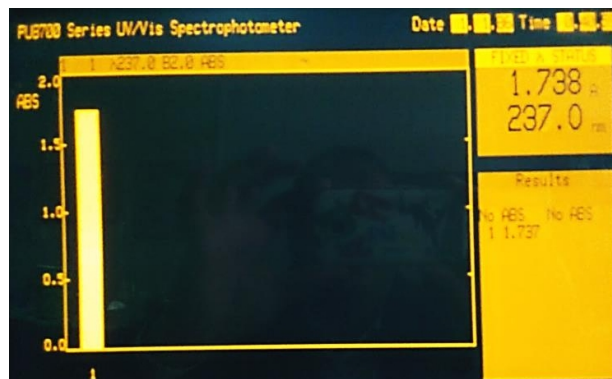


Figure 7: Absorbance peaks level for Standard vit.D2 using UV-VIS at Representative wavelength (Fix λ = 237 nm)

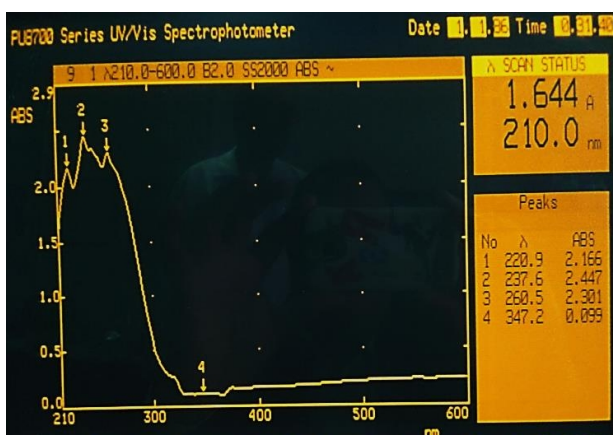


Figure 6: Absorbance peaks level for *C. Roseus* Alcoholic extract by UV-VIS

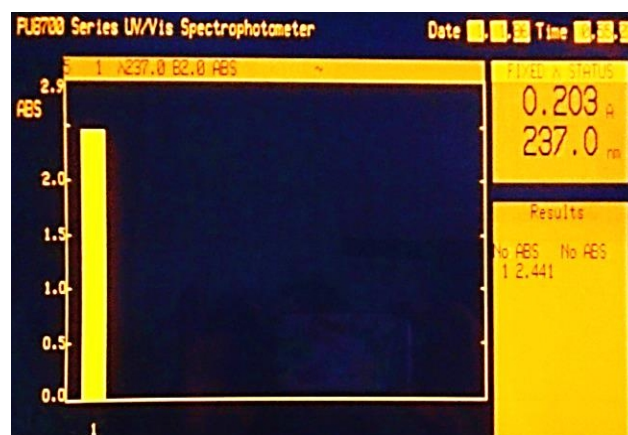


Figure 8 : represents absorbance peaks level for alcoholic extract of *C. roseus* using UV-VIS at Representative wavelength (Fix λ = 237 nm)

Preparation of standard curve using a spectrophotometer (UV-VIS)

Standard curve was plotted from analysis of standard vitamin D2 in order to measure Vitamin D2 concentration exist in alcoholic extract of periwinkle plant; this process was done through simultaneous

preparation for several concentrations of standard material (Ergocalciferol). Prepared concentrations values was (1, 2, 3, 4 IU/gm) diluted from the original standard its concentration (100,000 IU / gm). Reading of standard solutions was done at representative wavelength 237 nm.

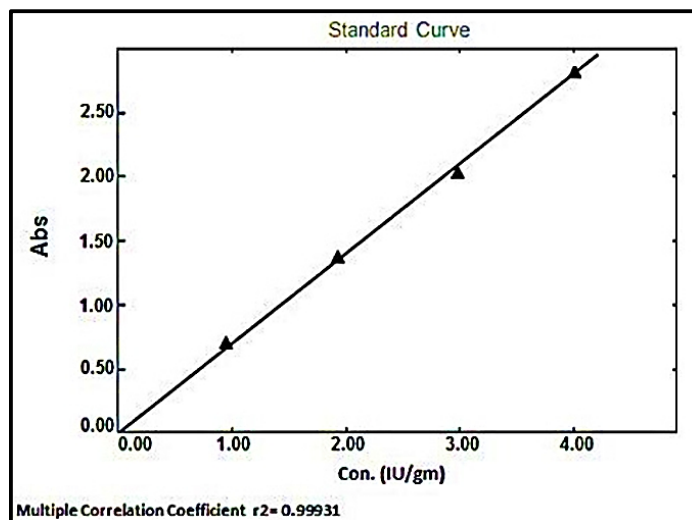


Figure 9: standard curve of vitamin D 2

Qualitative Identification of Vit.D2 in *C. Roseus* using Infrared instrument

Two separately transparent disks of standard Vit.D2 and dry grinded periwinkle plant was prepared for the purpose of examine by infrared instrument. This process has been done through grinding each material separately with potassium bromide, then compress the mixture by

piston under very high pressure in the form of a transparent disk. These two disks were examined using infrared instrument, the spectrum results are shown in Figures 10, 11, 12, which represents (transmittance T%) of effective groups for standard Vit.D2 and dry grinded plant respectively using IR-Prestige instrument [14].

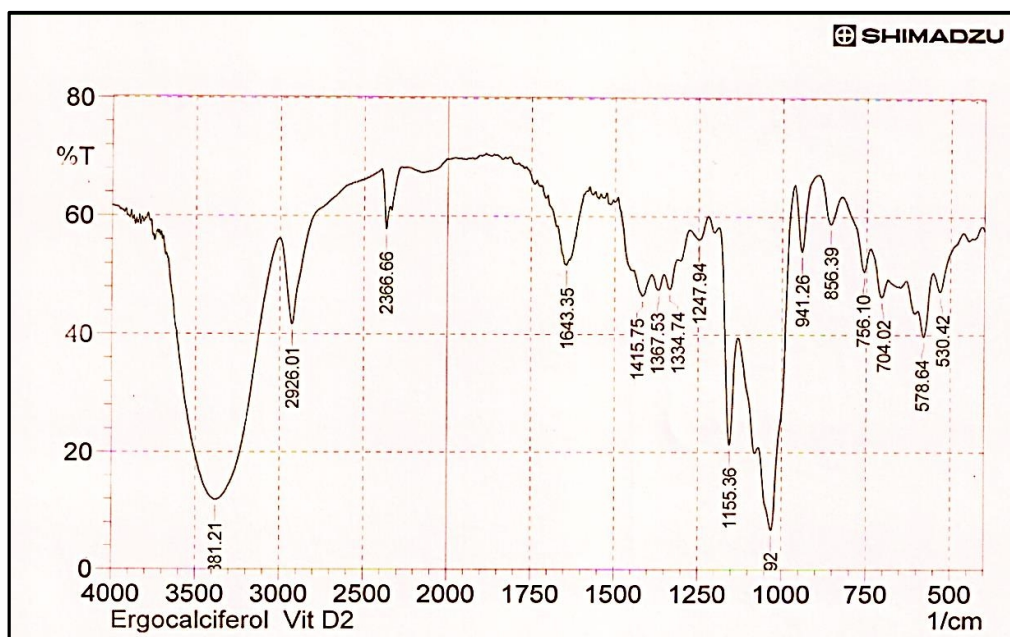


Figure 10: represents (transmittance T%) for effective groups of standard vitamin D2 in IR-Prestige instrument

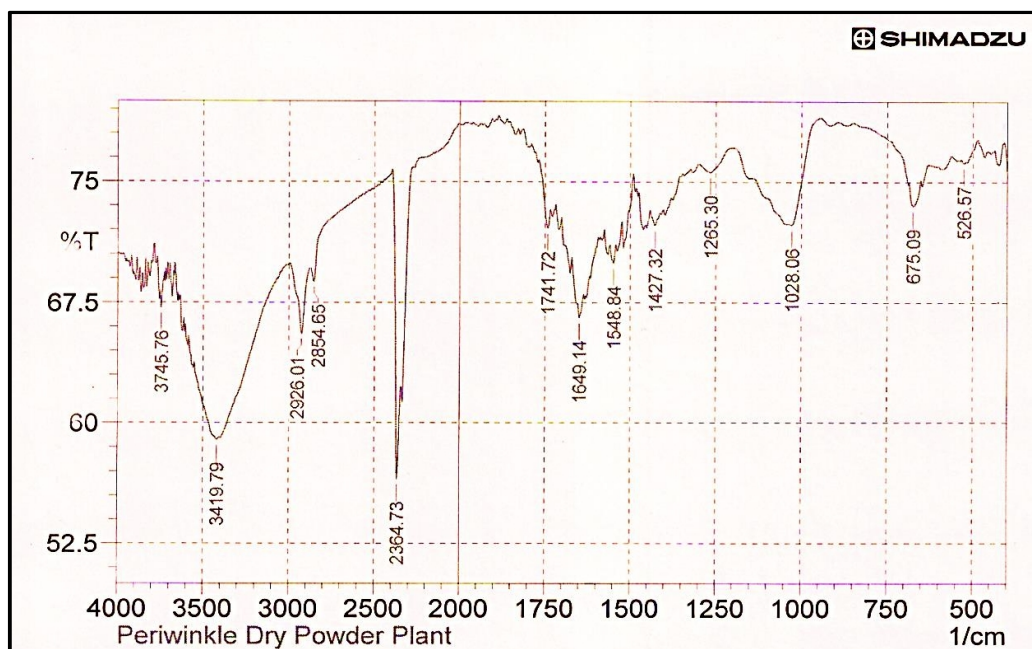


Figure 11 : represents (transmittance T%) for effective groups of dry grinded C. Roseus using IR-Prestige instrument

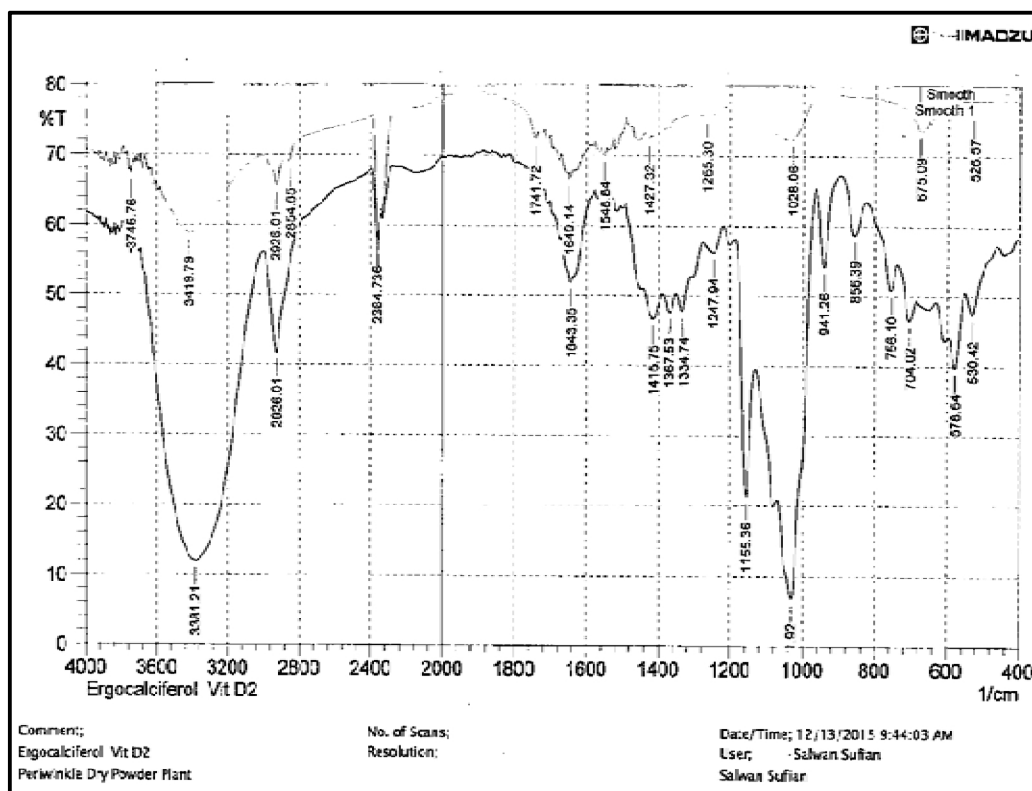


Figure 12: represents matching molecular spectra (transmittance T%) for effective groups of standard vitamin D2 and dry grinded periwinkle plant using IR-Prestige instrument

Qualitative Identification of Vit.D2 in *C. Roseus* using ATR-FTIR

A new approach requiring minimal sample preparation for the quantitative and qualitative analysis components has been investigated utilizing attenuated total internal reflectance infrared spectroscopy (ATR-FTIR). This technique is used to

examine and analyze all types of samples as they are, whether solid, liquid, powders, pastes, pellets, slurries, fibers, and others. Samples placed directly on the accessory crystal for examine and analysis in which intensity graphic of permeability appear within seconds as it shown in figures 13 and 14 [20].

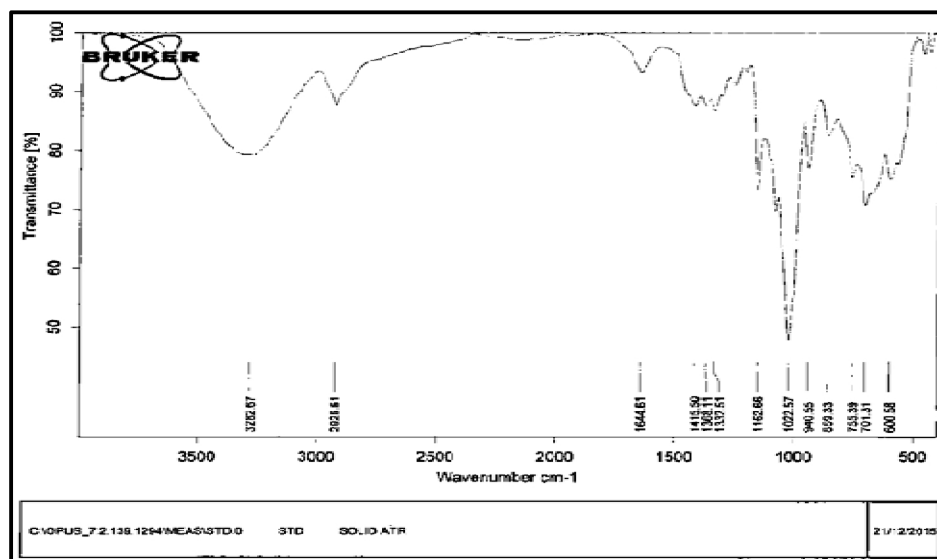


Figure 13: represents molecular spectra (transmittance T%) of effective groups for Standard vit.D2 using ATR-FTIR

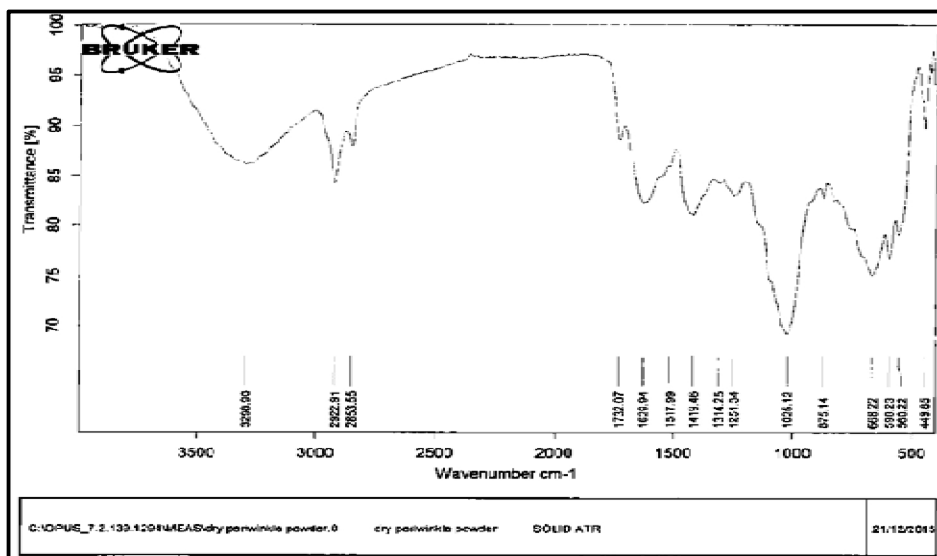


Figure 14: represents molecular spectra (transmittance T%) of effective groups for dry grinded *C. roseus* using ATR-FTIR

Results

Concentration calculations resulting from HPLC

1. Using area under the peak -obtained and shown in figures 3,4- to calculate the aqueous extract concentration by the following equation: -

$$C_{\text{sample}} = \frac{C_{\text{standard}} \times A_{\text{sample}}}{A_{\text{standard}}}$$

Sample con. (IU/gm) = (Con. standard X Area of sample) / Area of standard

$$= (2.5 \times 2683983) / 357216 \\ = 18.784 \text{ IU/gm Vit.D2 in aqueous extract only}$$

2. Calculation of vit. D2 concentration in original solid state of *C. roseus* plant by the following equation:-

$$C_{\text{solid sample}} = \frac{C_{\text{aqueous extract}} \times V_{\text{aqueous extract}}}{Wt_{\text{sample}}}$$

Sample con. (IU/gm) = (Con. of the aqueous extract X volume of aqueous extract) / intake sample weight

$$= (18.784 \times 500) / 50 \\ = 187.840 \text{ IU/gm Vit.D2 in drying weight of } C. \text{ roseus,}$$

Determination of Vit.D2, and concentration calculations of alcoholic extract of *C. roseus* resulting from (UV-VIS)

Using the calibration curve, straight line equation, and readout device for standard Vit.D2 was (1.737) as shown in Figure (7). Absorbance value of aqueous extract sample was (2.441) as shown in figures (8). concentration obtain from the instrument was (1.723) which represents the alcoholic extract concentration only. The concentration of Vit. D2 in the original solid state of the plant can be obtained from the following law:-

$$\text{Vit.D2 con.in solid state (IU/gm)} = (\text{Con. Of alcoholic extract X volume of alcoholic extract X dilution factor}) / \text{intake sample weight gm} \\ = (1.723 \times 250 \times 10) / 25 = 172.3 \text{ IU/gm}$$

Discussion

Results shows that conditions of separation and analysis methods were applied successfully, very efficient, and accurate for Vit.D2 determination. Quantitative results and mathematical calculations obtained from HPLC shows that *C. roseus* contain good acceptable concentration of this vitamin up to 187.840 IU/gm in drying weight by applying the special equations of this technique using the concentration, area of standard material and area of sample.

HPLC is one of the most important comparing techniques between samples and standard material. Qualitative analysis shows the appearance chromatogram peak for standard material, sample at very closely retention time of (3.026, 2.871 min) respectively; indicate that these analyst compounds have the same molecular weight, physical, and chemical properties. Thus these analyst compounds are same.

Qualitative analysis obtained from UV-VIS spectrophotometer shows match and closely (λ max for 3 chromatogram peaks respectively) at specific wavelength ranged from 210 to 275 nm for effective groups of analyst *C. roseus* and standard material as it shown in figures 5 and 6.

Qualitative analysis obtained from IR-Spectrophotometer and FTIR shows match spectra of standard material and sample at very closely wave number as it shown in figures (10,11,12,13,14). These spectra indicate that these analyst compounds have the same effective groups as follows; O-H groups ranged from 3700-3500 cm^{-1} , C-H stretch ranged from 2900-2800 cm^{-1} , C-C stretch aromatic ring ranged from 1500-1400 cm^{-1} , =C-H bend ranged from 1000-600 cm^{-1} .

Conclusions

The present study concludes that results of detection and analysis confirmed the existence and containing high concentration of vitamin D2 in *C. Roseus*. Since the possibility of transplant it easily; so it is possible to obtain very large amount of this plant and then getting vitamin D2 to using it in industrial scale after extracted,

isolated and purified for use in medical, pharmaceutical fields and alternative medicine, or what is called herbal medicine. There for there is a need for natural resources such as plants sources instead of chemical due to low or no side effects. Four equipment's at least; used to confirmed the presence and quantity Vit. D2 in *C. roseus*, and it is the first record in Iraq. It's the first step to confirm many facts that deal with natural plants due to medicinal activity.

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