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Qualitative and Quantitative Determination of Anti-Cancer Drug (Vincristine) in *Catharanthus Roseus* by High Performance Liquid Chromatography and Qualitative Identification using other Molecular Spectra Instruments.

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ABSTRACT

SHIMADZU Preparative-High-Performance Liquid Chromatography was used for qualitative and quantitative estimation of vincristine in aqueous extracted of *Catharanthus Roseus* plant; (20µl, 2µg/ml)standard vincristine 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. Conditions of separation were; acetonitrile: phosphate buffer: 0.02M sodium dihydrogen phosphate (20:75:5 ml) as isocratic mobile phase, column C18-ODS (25cm x 4.6 mm x 5µm), 20 µl injection volume of sample, and flow rate 1.2 ml/min at 30ºC through 254 nm wave length UV detector Spectrophotometer. An intensive analysis conducted on aqueous, alcoholic extract and dry powder of plant leaves using modern and sophisticated instruments. Alcoholic extraction used for analysis by UV-VIS Spectrophotometer; transparent disk of potassium bromide and dry powder of plant leaves used for analysis using Infra-Red, while dry powder of *Catharanthus Roseus* leaves used for direct analysis using Attenuated Total Reflection - Fourier Transform Infrared Spectroscopy.Quantitative results obtained from HPLC shows that *C. Roseus* contain good acceptable concentration of vincristine drug up to 148 µg/gm. Qualitative results obtained from HPLC, UV-VIS, IR, ATR-FTIR shows match the effective groups of pure standard vincristine and alcoholic extract, dry powder of C. Roseus plant.

Keywords: *Catharanthus Roseus*, Vincristine, Pre-HPLC, Molecular Spectra Instrument.

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INTRODUCTION

Plants are conceders as one of the most important sources for drugs and medicines manufacture in which they contain high values of chemical, 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 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 which called alternative medicine and became it's own medications called Herbal Medical Therapeutics, thus become one of the remedies for many disease [1].

*Catharanthus Roseus*is one of the most important medicinal plants in the world. Botanical genus of Apocyanaceae Family, which includes five types. The scientific name of the plant is (*Catharanthus Roseus*) and English name is (*Periwinkle*) [2].Flowering circular shape composed of five petals and are several colors, including white, red, pink and purple, native to Madagascar's original south-east and east, and the plant is grown easily [2-4].Image No. (1) represent *Catharanthus Roseus* in nature. Now it is grown in almost worldwide [5], has been used periwinkle plant in Madagascar, and in many countries a later time, a popular diabetes therapy, blood pressure, asthma, constipation, liver disease, kidney disease, cancer, and problems with menstruation [2,4].

Image No. (1) *Catharanthus Roseus* in nature

Periwinkle plant contains many important compounds such as Indole alkaloids, carbohydrates, saponin, Tannin, phenols and flavonoids, which are plants' derivatives from multiple hydroxyl and glycosides flavonoid derivatives. Alkaloids are the 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].

Vincristine (VCR)

Is a natural alkaloid belongs to a group of bisindole alkaloids derived from *Tryptophan* with molecular formula ($C_{46}H_{56}N_4O_{10}$.), molecular Weight (824.97) found and synthesized in (*C. Roseus*). VCR is an anti-neoplastic drug isolated from this plant. Vincristine was approved by the Food and Drug Administration (FDA) in 1984. It was formerly known as Leurocristine, Oncovin, Vincasar, and Vincrex. Sometimes abbreviated "VCR". VCR is a lipophilic amine, first introduced as anticancer chemotherapy over 45 years ago. Clinically it is used to treat a range of cancers including leukemia[6].Vincristine is on the World Health Organization's List of Essential Medicines, a list of the most important medication needed in a basic health system[7]. Figure (1) Vincristine Structure and active groups.

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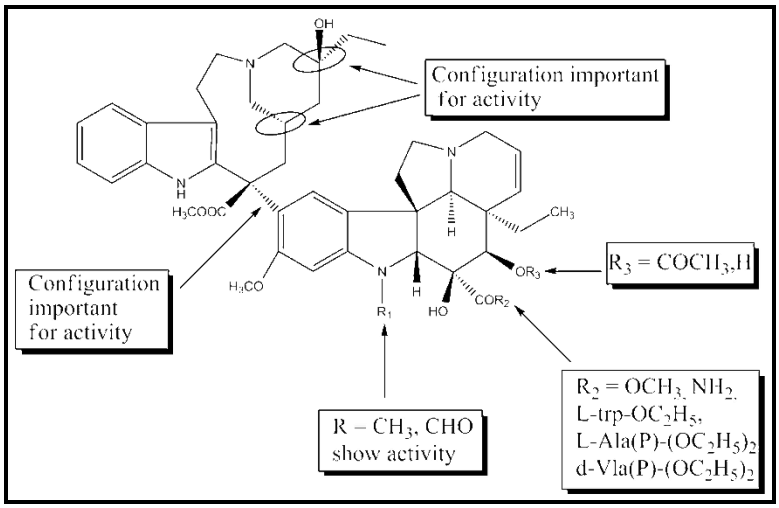


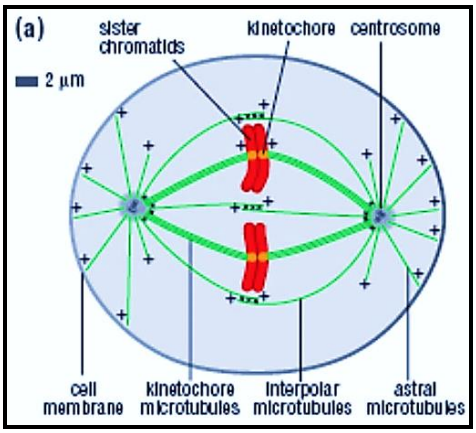
Figure (1) Vincristine Structure and active groups

Biosynthesis of Vincristine

Vincristine belongs to a group of alkaloids that derive from tryptophan. The structure of vincristine is derived by coupling of two alkaloids, catharanthine and vindoline. The biosynthesis of vincristine is summarized as follows; First, catharanthine (1) is oxidized by a peroxidase catalyst (2), which forms a peroxide which acts as a leaving group. When the peroxide leaves, the carbon-carbon bond is broken and the intermediate electrophilic ion (3) is attacked by the nucleophilic vindoline (4). The molecule is then reduced in the dihydropyridinium ring by NADH-dependent 1,4-addition, giving the substrate for hydroxylation (7). Finally, reduction by NADH yields vincristine [8].

Vincristine Mechanism Action

VCR is a cell cycle-dependent compound that directly and irreversibly binds to microtubules and spindle proteins in S phase of the cell cycle and interferes with the formation of the mitotic spindle, binds to tubulin, causing microtubule depolymerization, M-phase arrest, and apoptosis in mitotic cells; thereby arresting tumor cells in metaphase. At low concentrations, VCR induces reversible mitotic arrest with little effect on morphology or polymerization of spindle microtubules. In contrast, higher VCR doses and long-term VCR exposure are associated with microtubule depolymerization-induced cytotoxicity [9,10]. Figure (2) Normal Microtubules, Figure (3) Arrested Microtubules.



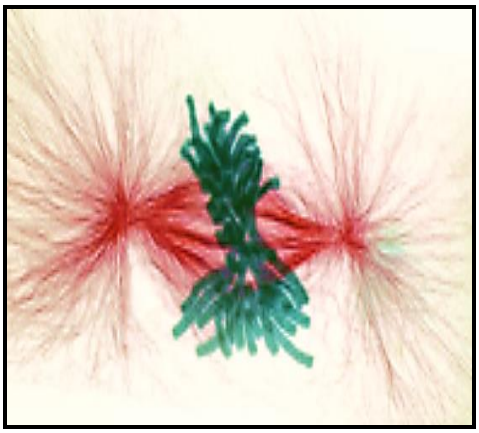


Figure (2) Normal Microtubules **Figure (3) Arrested Microtubules**

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MATERIALS AND METHODS

Materials

A. **Plant:** *C. Roseus*plant samples were collected freshly from local markets in Baghdad city. Samples washed by tap water to get rid of impurities and dust, and then washed again with distilled water. After that samples were stored at (-20ºC) in pvc bags until time of analysis.

B. Chemicals: Chemicals that have been used in the present study obtained from thoughtful international companies were high purified as follows; acetonitrile, methanol, ethanol, and distilled water for HPLC. Another chemicals were used in this study as follows; standard vincristine, tartaric acid, ethyl acetate, sodium dihydrogen phosphate, and ortho-phosphoric acid. Potassium bromide powder has been used for preparation of Infra-Red transparent disk**.**

C. 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.

Methods:-

First:-

A. Detection, Qualitative and Quantitative Determination of Vincristine in *C. Roseus* Using RP-HPLC

Standard material and Samples were analyzed by High Performance Liquid Chromatography (HPLC) system, SHIMADZU model 10AV-LC equipped with binary delivery pump model LC-10AV, the eluted peaks were monitored by UV-VIS detector SPD-20A. The condition of separation are listed in table (1) [11].

Parameter	Characteristic for VCR identification
Mobile phase	Acetonitrile: phosphate buffer: 0.02 M sodium dihydrogen phosphate (20 :75 : 5 ml)
Column Type	C18-ODS (25cm x 4.6 mm X 5µm)
Volume injection sample	20 µl
Detector	UV Spectrophotometer at 254 nm
Flow Rate	1.2 ml / min
Temperature	30ºC

Table (1): Separation conditions of High performance liquid chromatography

B. Preparation and analysis of standard solution

1. 2 µg/ml prepared by dilution from original pure standard vincristine (1mg/ml).
2. 20 µl of standard was injected into HPLC.
3. Obtaining results that contain the peak top, retention time, and area under the peak for standard as it shown in Figure (4).
4. Make the necessary calculations.

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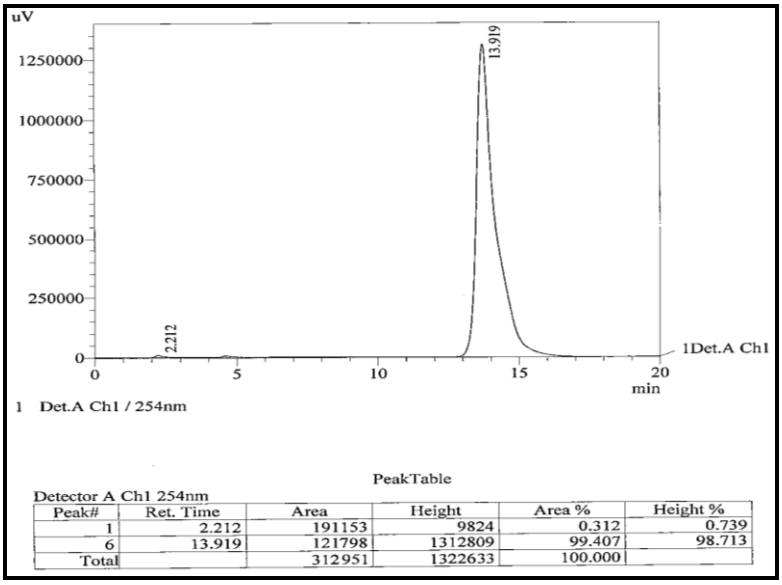


PeakTable

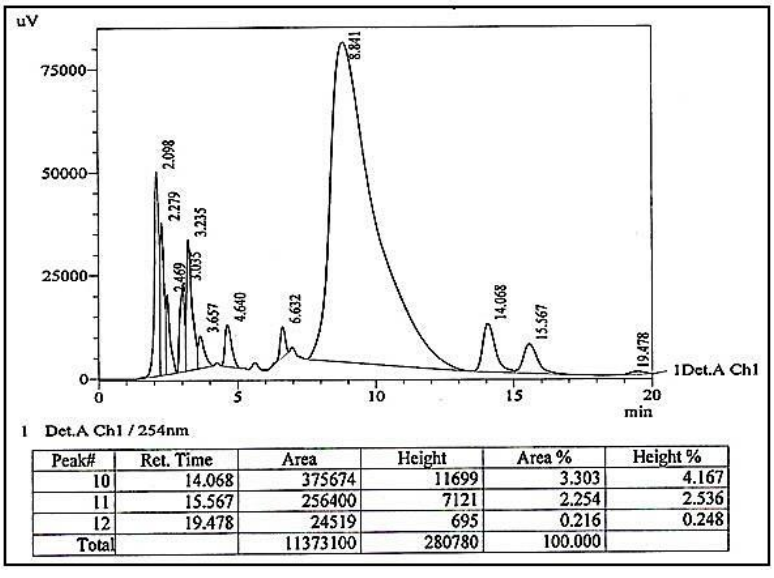
Detector A Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.212	191153	9824	0.312	0.739
6	13.919	121798	1312809	99.407	98.713
Total		312951	1322633	100.000	

Figure (4): chromatogram of peak top when (20µl, 2µg/ml) standard vincristine injected into HPLC

C. Aqueous extraction:-detection and estimation of vincristine in *C. Roseus* plant using (RP-HPLC) [11].

1. Weight 50 g of stems, leaves, and flowers, and then placed directly in electric blender.
2. Addition of 1.2 L ml of distilled water and mixed for 5 minutes.
3. Sample placed on magnetic stirrer for 72 hours at 37ºC. Sample must be well covered to protect from oxidation and prevent conversion by ultraviolet radiation of sun light.
4. Sample filtration by Buechner funnel and passing into activated charcoal.
5. Filtration by Whatman filter Paper 0.45µm and Millipore filter (0.22 µm).
6. 20µL of aqueous extract sample was injected into (RP-HPLC).
7. Obtaining results that contain the peak top, retention time, and area under the peak for sample as it shown in Figure (5).



Peak#	Ret. Time	Area	Height	Area %	Height %
10	14.068	375674	11699	3.303	4.167
11	15.567	256400	7121	2.254	2.536
12	19.478	24519	695	0.216	0.248
Total		11373100	280780	100.000	

Figure (5): chromatogram of peak top when (20µL) plant aqueous extract sample injected into HPLC

ISSN: 0975-8585

Second:-Qualitative Detection of Vincristine in *C. Roseus* by(UV-VIS) [1, 12]

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

Sample preparation and extraction

1. Plant samples dried in shade at room temperature for a week within well-ventilated environment, and turning plant parts to avoid fungal growth.
2. Grind the dry parts of the plant (stem, leaves, and flowers) by ceramic mortar.
3. Weighting (25 gm) of ground dry plant and placed into thumble.
4. Addition of (250 ml) ethanol into round bottom flask.
5. Soxholet escalation for (12 hours) at 80ºC.
6. Extract evaporating by rotary evaporator, then analysis by UV-VIS.
7. Transfer the sample to freeze dryer to conversion it into powder for other tests. Figure (6,7) represents absorbance peaks level for standard vincristine, and plant alcoholic extract respectively using UV-VIS spectrophotometer.

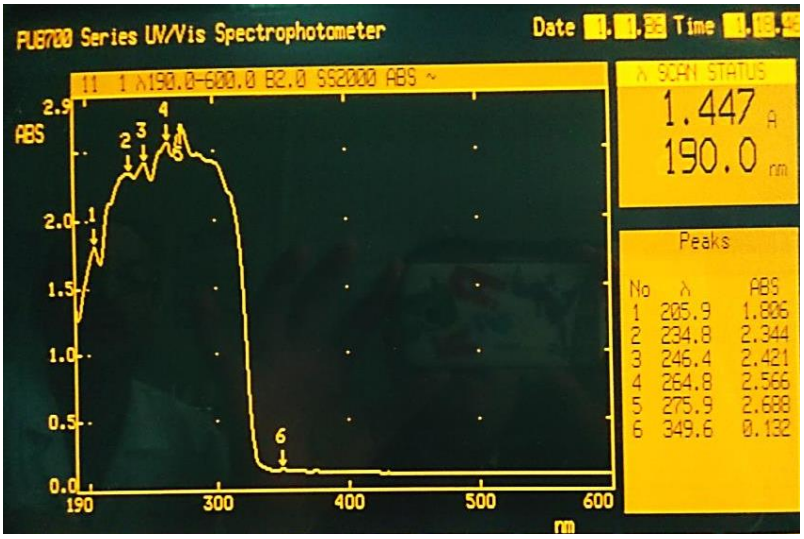


Figure (6) represents absorbance peaks level for standard vincristine using UV-VIS

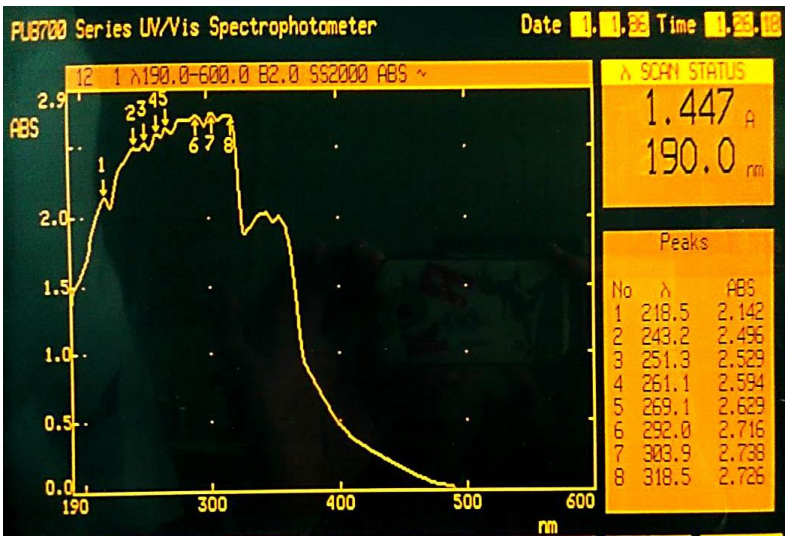


Figure (7) represents absorbance peaks level for plant alcoholic extract using UV-VIS

ISSN: 0975-8585

Third:-**Qualitative Detection of Vincristine in *C. Roseus* by Infrared instrument**

Two separately transparent disks of standard VCR 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 Figure (8,9) which represents (transmittance T%) of effective groups for standard vincristine, and dry grinded plant respectively using IR-Prestige instrument**[13]**.

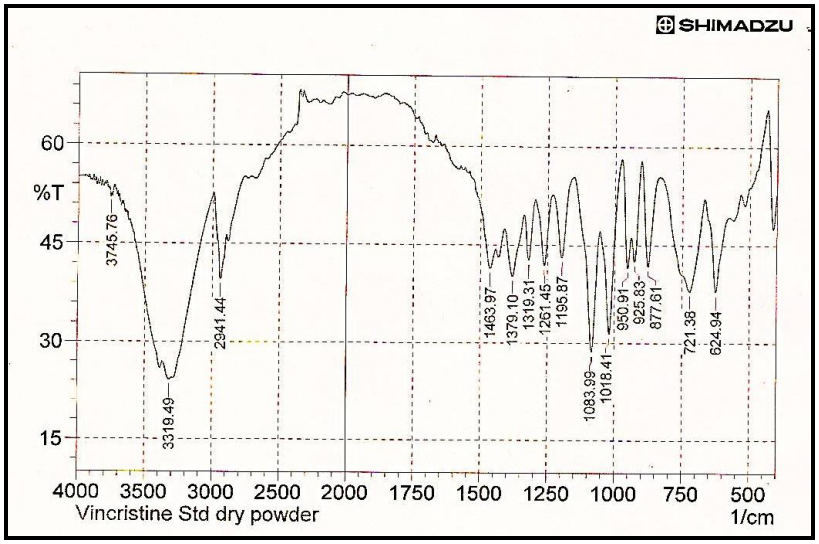


Figure (8) represents (transmittance T%) for standard Vincristine using IR-Prestige instrument

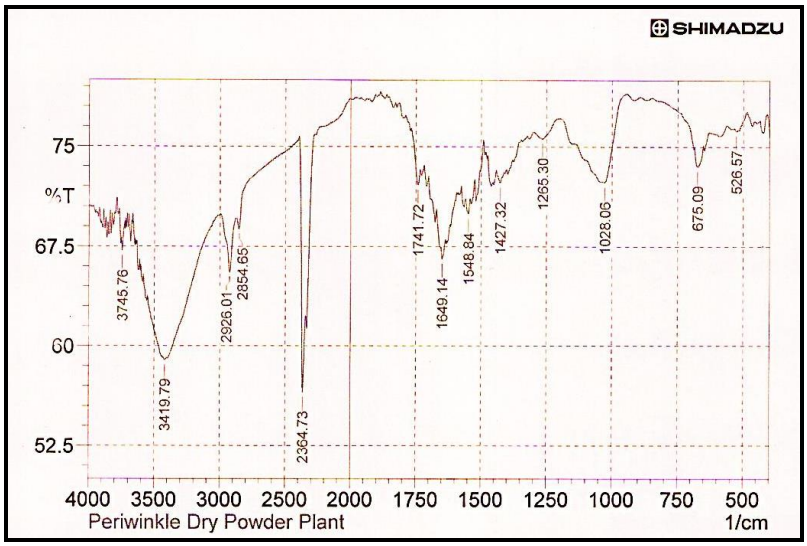


Figure (9) represents (transmittance T%) for effective groups for dry grinded plant using IR-Prestige instrument

Fourth:-**Qualitative Detection of Vincristine in *C. Roseus* by ATR-FTIR**

Attenuated Total Reflection - Fourier Transform Infrared Spectroscopy

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

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examine and analysis in which intensity graphic of permeability appear within few seconds as it shown in Figure (10,11) which represents the molecular spectra (transmittance T%) of effective groups for standard vincristine, and dry grinded plant respectively using ATR-FTIR instrument**[14]**.

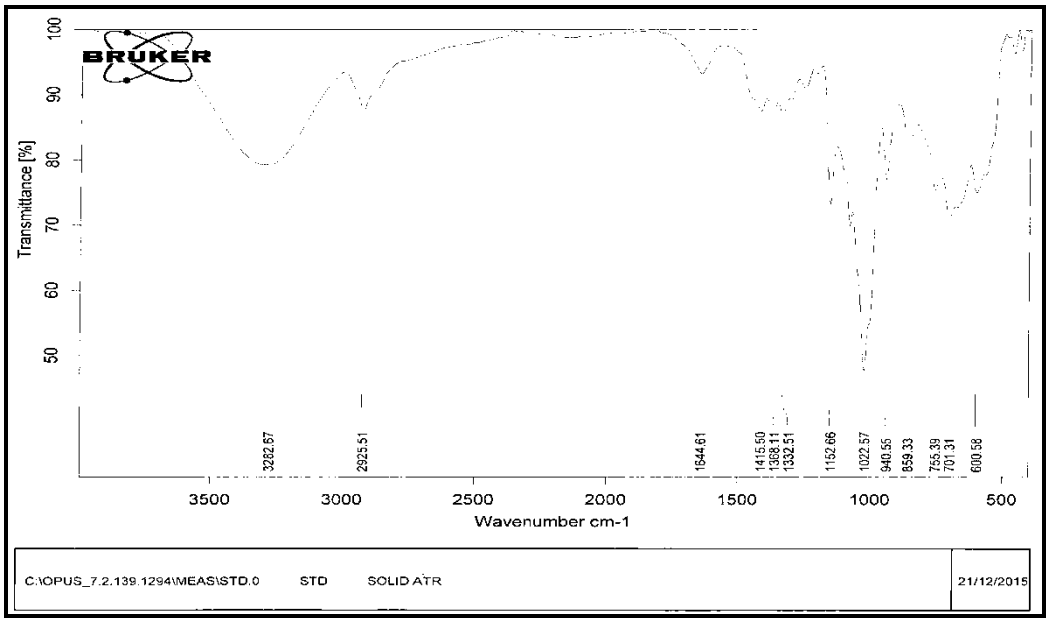

Figure (10) represents the molecular spectra (transmittance T%) of effective groups for standard vincristine using ATR-FTIR instrument

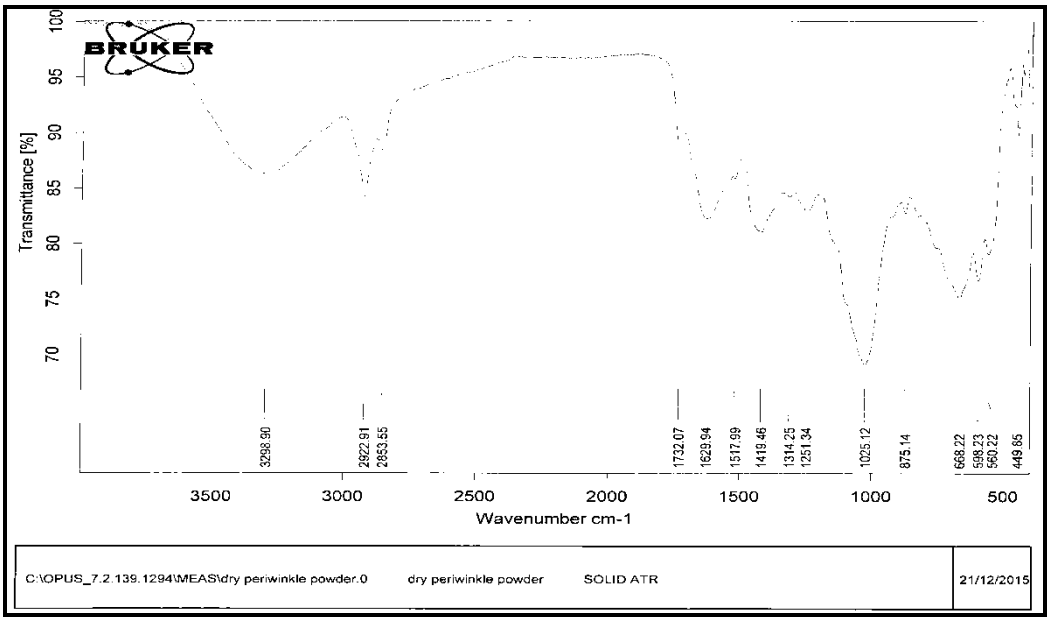

Figure (11) represents the molecular spectra (transmittance T%) of effective groups for dry grinded plant using ATR-FTIR instrument

ISSN: 0975-8585

RESULTS AND DISCUSSION

Concentration calculations resulting from HPLC

$$C_{sample} = \frac{C_{standard} \ X \ A_{sample}}{A_{standard}}$$

Using area under the peak to calculate the aqueous extract concentration by the following equation: -

Sample con.(µg/ml) = (standard con. X area of sample) / area of standard
= (2 µg/mlX 375674) / 121798
= 6.168 µg/ml vincristine in aqueous extract

Concentration calculation of vincristine in original solid state of the plant by the following equation:-

$$C_{sample} = \frac{C_{standard} \ X \ A_{sample}}{A_{standard}} X \frac{D.F}{Wt. \text{ or } V}$$

= (6.168 µg/ml X 1200 ml) / 50gm
= 148.032 µg/gm vincristine in original plant

DISCUSSION

Results shows that conditions of separation and analysis methods were applied successfully, very efficient, and accurate for vincristine determination. Quantitative results and mathematical calculations obtained from **HPLC** shows that *C. Roseus* contain good acceptable concentration of vincristine drug up to 148 µg/gm by applying the special equations of this technique by using the concentration, area of standard material and area of sample.

High-Performance Liquid Chromatography 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 (13.919, 14.068 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 5 chromatogram peaks respectively) at specific wavelength ranged from(205 to 275 nm) for effective groups of analyst *C. Roseus* and standard material as it shown in figs. 6,7.

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 (8,9,10,11). These spectra indicate that these analyst compounds have the same effective groups as follows; O-H groups ranged from 3700-3500 cm^{-1}, N-H groups ranged from 3500-3300 cm^{-1},C-H stretch ranged from 2900-2800 cm^{-1}, C=N stretch ranged from 2360-2310 cm^{-1},C=O stretch ranged from 1750-1650 cm^{-1}, C-C stretch aromatic ring ranged from 1500-1400 cm^{-1}, C-N stretch aromatic amines ranged from 1260-1250 cm^{-1}, C-N stretch aliphatic amines ranged from 1250-1000 cm^{-1}, =C-H bend ranged from 1000-600 cm^{-1}.

CONCLUSIONS

Vinca alkaloids possess beneficial properties such as slow cancer cell growth. Vincristine is a very interesting and useful drug, synthesized from catharanthine and vindoline through the alkaloid pathway, it is used to treat various cancers by disrupting the formation of microtubules cells, which inhibits the replication of cancer cells. Since vincristine expresses the lowest risk of peripheral neuropathy out of the four Vinca alkaloids, the positive effects outweigh the negative. Vincristine is a useful drug used to treat various lymphomas and sarcomas, advanced testicular cancer, breast cancer, acute leukemia, neuroblastoma, and other types of cancers. The present study concludes that results of detection and analysis confirmed the existence and containing good acceptable concentration of anti-cancer drug (vincristine) in *C. Roseus*. In Iraq, to the best of our knowledge, this is the first study for isolation, purification, identification and determination of this drug using multiple scientific instruments. These results obtained by modification, development of analysis methods and adjustment of some analysis conditions. Since the possibility to transplant *C. Roseus* plant easily, it is possible to obtain very large amount of this plant and then getting vincristine for using in industrial scale after extracted, isolated and purified to use in medical, pharmaceutical fields and alternative medicine, or what is called herbal medicine.

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REFERENCES

[1] Abd-Alwahab A. Technology of Applied of Sciences, Biotechnology Division 2010; 8, 20-26.
[2] Sain M. Sharma V. International Journal of Pure & Applied Bioscience 2013; 1 (6) 139-142.
[3] TG Tutin, VH Heywood, NA Burges, DM Moore, DH Valentine, SM Walters and DA Webb. Cambridge University Press. 2010; 3 4th Edition.
[4] A Bakrudeen, G Subha Shanthi, T Gouthaman, M Kavitha, M Rao. Acta Botanica Hungarica 2011; 53 (1-2) 197-209.
[5] Nejat N, Valdiani A, Cahill D, Tan YH, Maziah M, Abiri R. Hindawi Publishing Corporation the Scientific World Journal. 2015; 1-19.
[6] Vincent Ting. The Occurrence, Bioactivity, Biosynthesis, and Synthesis of Vincristine. Chemistry 150, Fall 2008. 1-3.
[7] "WHO Model List of Essential Medicines". World Health Organization 2014.
[8] Harvey MJ, Banwell MG, Lupton DW. Tetrahedron Letters. 2008; 49, 4780-4783.
[9] V Sravanthi, VK Mayure, GJ Finny, CP Meher. Pharma Tutor. 2014; 2(8); 119-153.
[10] Jordan MA. Current Medicinal Chemistry - Anti-Cancer Agents. Bentham Science 2015; 2. 1.
[11] Kevin J Len, Helene Ther, J Richard Wag. Journal of Chromatography. 2010; B. 877: 3400–3404.
[12] Ibrahim M, Mehjabeen SS, Narsu ML. International Journal of Pharmaceutical Applications. 2011; 2, 3. 165-173.
[13] Mohammadi M, Ghanbarzadeh B, Hamishehkar H. Advanced Pharmaceutical Bulletin 2014; 4(2) 569-575.
[14] Szabo L, Bolcskei H, Eszter B, Mak M, Szantay C. Pharmaceutical & Medicinal Chemistry 2001; 334 (12), 339—405.