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Overview of Vinblastine Extraction from Catharanthus Roseus using the Supercritical Fluid Extraction Technique

Pratyusa Mandal[1], Saumya Dixit[2], Ankan Ghosh[3], Abhirup Ghosh[4], Saptrishi Ghosh[5]

[1-5]Student, Department of Bachelor of Technology in Biotechnology, School of Biosciences and Technology, Vellore Institute of Technology, Vellore-632014

Abstract: *Vinblastine (VBL), an alkaloid derived from the plant Catharanthus roseus has been found to be a drug of paramount importance as it is serving as one of the major chemotherapy drugs that are available in today's market. VBL is sold under the brand name Velban and is useful in treating a number of diseases like non-small cell lung cancer, Hodgkin's lymphoma, various types of carcinomas and sarcomas, melanoma, uveal melanoma, and diseases like AIDS-KS, etc. VBL was first isolated in 1958 and used as herbal medicine. Due to the increased need, the production of vinblastine had to be scaled up. In this article, we have reviewed various articles available regarding VBL extraction, purification, analysis, and quantification. This article focuses primarily on the supercritical fluid extraction (SFE) method, the description of the method, the instrumentations, applications, advantages, and disadvantages. The quantitative analysis of the extract is performed using UV-equipped high-performance liquid chromatography (HPLC) and mass spectroscopy (MS). In the purification step, thin layer chromatography (TLC) is used.To improve the efficiency of the SFE procedure, we have suggested some improvements and optimization by analyzing the effects of different parameters like temperature, pressure, co-solvent, and raw materials.*

***Key Words: Catharanthus roseus*, vinblastine, supercritical fluid extraction, quantitative analysis, purification, optimization, carbon dioxide, ethanol**

1. INTRODUCTION

Vinblastine is a natural alkaloid isolated from the plant *Catharanthus roseus Linn* and is the chemical analog of vincristine. Vinblastine has a molecular formula $C_{46}H_{58}N_4O_9$. Vinblastine tends to bind to tubulin and inhibit the formation of the microtubule, which results in disruption of mitotic spindle assembly and arrest the tumor cells in the mitotic phase of the cell cycle, all of these processes are necessary for the separation of chromosomes during anaphase of mitosis [1]. Amino acid, cyclic AMP, and glutathione metabolism can get affected when VBL interferes in the process [2]. At very low concentrations VBL suppresses the microtubule dynamics and at higher concentrations, it reduces microtubule polymer mass [3]. The elimination half-life of vinblastine is approximately 29 hours and a very little amount of drug remains in our body at 48 hours [4]. Vinblastine is also used for medicinal purposes such as a number of chemotherapy regimens that include ABVD for Hodgkin's lymphoma and also has some protocol to treat histiocytosis. Its first extraction and purification using organic solvents methods were patented by the pharmaceutical company Eli Lilly in the 1970s [5], [6].

Supercritical fluid extraction (SFE) is the process of separating individual constituents from a mixture with the help of extracting solvent which is a supercritical fluid such as supercritical carbon dioxide [7]. The matrix from which the extraction is done is usually a solid one, but in certain cases, it can be a liquid matrix as well.

The objectives of SFE are extracting minute amounts of the desired product from large samples, purifying the desired product from unwanted materials, or sample formation for analytical purposes [6]. The most preferred supercritical fluid is carbon dioxide, which is often used in combinations with co-solvents such as ethanol or methanol. The ideal conditions for supercritical carbon dioxide extraction, to get high-value products from natural materials, are a temperature and pressure above 31°C and 74 bar. To slightly vary these conditions, certain modifiers can be used [8]. Interestingly, the extraction process leaves no solvent residue behind. Due to its low critical temperature of 31°C, carbon dioxide is known to be perfectly adapted in food, aromas, essential oils, and nutraceutical industries [10].

Supercritical fluid carbon dioxide can best be imaged as a dense fog when it is used in a dense liquid state. Low-pressure carbon dioxide is often the most suitable method for producing high-quality botanical extracts [10].

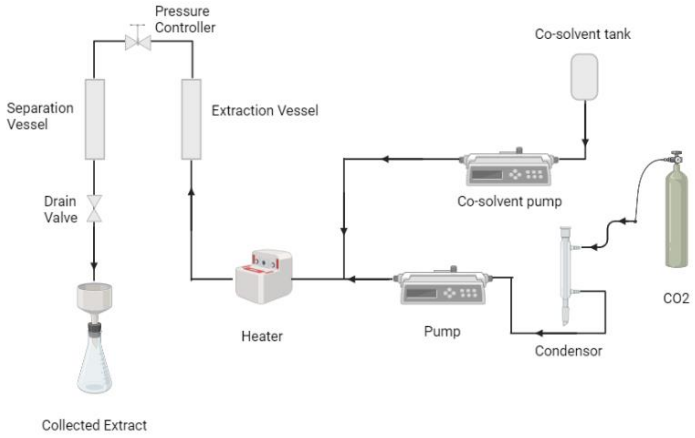

Fig-1: Schematic diagram of the experimental setup for supercritical carbon-dioxide extraction [9]

2. APPLICATIONS

- The main secondary metabolites of *C. roseus* are terpene indole alkaloids (TIAs) with important applications in human medicine which are presenting biological activities such as antitumor, anti-diabetes, anti-helminthic, antihypertensive, anti-diarrhea, antimicrobial actions, and others [11].

- Vinblastine is one of the major anti-cancer drugs. Anti-cancer drugs act via mitotic arrest. They disengage the spindle fibers formed during the metaphase stage of mitosis by denaturing the spindle fiber proteins, thus arresting mitosis. As a result, the cell may go through unequal division, death, or exit without division [12].

- The major application of VBL in the medical industry is to treat certain cancers such as Hodgkin lymphoma, non-Hodgkin's lymphoma, soft tissue sarcoma, Kaposi's sarcoma, testicular cancer, breast cancer, lung cancer (non-small cell lung cancer), head and neck cancer, and bladder cancers, melanoma, mycosis fungoides (t-cell lymphoma), and choriocarcinoma. Germ cell tumors and fibromatosis can be cured by using VBL [2].

- Histiocytosis, a type of blood disorder, can also be treated by administering vinblastine.

- Despite being a very useful drug, vinblastine has some side effects like fatigue, weakness, low blood count, nausea, hair loss, fever, etc [13].

3. EXTRACTION PROCEDURE

The alkaloid in the plant is present in minute quantities, hence its isolation by techniques such as organic solvents methods, solid-liquid extraction, and column chromatography on a large scale is complex and time-consuming with several steps which lead to the generation of large amounts of organic waste, losing some volatile compounds, degrading thermolabile compounds and hence high production cost [14].

Thus, supercritical fluid extraction provides an alternative to traditional extraction processes of biochemical and pharmaceutical products and is one of the techniques preferred for the extraction and separation of non-volatile compounds since it is a non-destructive method for isolating minimal amounts of constituents from natural materials, higher selectivity, diffusivity, and ecology. In 1822, Baron Charles Cagniard de la Tour was the first person to discover the SFE technique, when he noticed changes in solvent behavior at a particular value of pressure and temperature [7]. The earliest known application of this technique was for the decaffeination of green coffee beans which started in Germany; and after a few years, the extraction of oils from hops using liquid CO_2 was developed in Australia [15]. As for the solvent, carbon dioxide is considered the most suitable since it is chemically inert, thrifty, affordable, separable from extracts, non-toxic, and is a sanctioned food-grade solvent [16]. The system uses CO_2 as a supercritical fluid and requires pumping of CO_2 with high pressure to contain the sample.

The components of SFE are the Fluid reservoir, pump, and extraction cell/column.

3.1 Materials

Sample preparation: Dried *C. roseus* leaves ground into powder

Chemicals: Carbon dioxide, ethanol, methanol

3.2 Supercritical Fluid Extraction Procedure

Supercritical fluid extraction of vinblastine is performed from *Catharanthus roseus*, using ethanol as a co-solvent for carbon dioxide in a continuous SFE system.

The carbon dioxide is supplied from a gas cylinder and directed to an electrically driven diaphragm-type compressor and thereafter filtered through filter discs. The extraction column is packed with glass beads and some amount of pre-treated powdered *C. roseus.* Pressurized supercritical carbon dioxide is made to flow through the column and it dissolves extractable compounds from the solid matrix [16]. A metal filter is inserted at the top of the column to reduce the entrainment of the sample and the

temperature within the air bath is controlled with the help of a proportional temperature controller. In the separator pressure reduction, temperature increase, or both, helps in the disassociation of the mixtures of extract and solvent after the dissolved compounds are transported to it by diffusing out [16]. Ethanol is added as a co-solvent (CO2/ethanol 2% w/w). To keep the pressure in the system constant, a back pressure regulator is deployed. The solute mixture of carbon dioxide leaving the top of the extractor is expanded to atmospheric pressure with the use of a micro metering valve. Through a Dry test meter and Flowmeter, the volume and the flow rate of carbon dioxide were measured. The temperature and pressure of the carbon dioxide are measured by the gauges equipped in the Dry test meter [16].

4. ADVANTAGES

Implementing SCF in the extraction process has benefits because it is environmentally friendly, it has health, chemical, and safety benefits as well.

- Most SCFs in industrial processes result from their replacement of far more environmentally damaging conventional organic solvents [17].
- Significantly low energy requirement.
- The reagents and fluids used are non-carcinogenic,
- non-toxic, non-mutagenic, and non-flammable.
- Supercritical fluids have high diffusivity, and low viscosity.
- The density and dielectric constant of SCFs can be altered by changes in operating pressure and temperature.

5. LIMITATIONS

- Requirement of high pressure which increases the cost.
- Carbon dioxide is a nonpolar gas, therefore having limited solubility.
- Limited range of materials that can be extracted [6].

6. QUANTITATIVE ANALYSIS

6.1 HPLC Analysis

The extract is evaporated under nitrogen till it dries completely and the residue is dissolved in methanol [18]. Samples are filtered through a non-sterile syringe filter unit and analyzed with an HPLC system equipped with a UV detector [19]. Chromatographic separations are carried out on an HPLC column. The mobile phase contains ammonium acetate buffer, acetonitrile, and methanol in varying proportions of 65:20:15 to 30:40:30 during a time period linearly gradient to a particular flow rate [20]. The analysis time varies with the amount of sample, and the elution of major alkaloids can be done before the whole sample is analyzed. The injections were made by an autosampler with an injection needle [21]. A computing system is used to analyze the collected data.

6.2 MS Analysis

Precursor ion scan is performed in positive ionization ESI mode from m/z 100-1000 for screening or detection of targeted TIAs [2]. Source-dependent parameters, ion spray voltage, source temperature (TEM), nebulizer gas, heater gas, and curtain gas are optimized at 5500 V, 550°C, 50 psi, 50 psi, and 20 psi, respectively [22], [23]. Nitrogen used as nebulizer, heater, curtain, collision-activated dissociation gas is set as medium and the interface heater is on. Each standard solution is taken separately in methanol for optimization of mass spectrometric conditions by direct infusion using a syringe pump. The most abundant fragment ions are selected for MRM transition [24].

7. PURIFICATION

During the process of extraction, many undesirable compounds get co-extracted with the target product, which results in an adulterated extract. Hence the case of crude vinblastine silica gel column chromatography is used for the purification process. After the extraction, the extract is loaded on a silica gel column. The silica gel is pre-equilibrated with chloroform and thereafter eluted with a gradient of chloroform: methanol [24]. The R_f values of different fractions on the chromatography column are noted. Fractions containing compounds that resemble the R_f value of standard vinblastine are pooled and incorporated to preparative TLC on a silica plate and developed in chloroform: methanol solvent system [25], [26]. The putative bands were scraped and eluted out with methanol. Thus, the purity is checked using TLC [27].

R_f= Distance travelled by the solvent / Distance travelled by the compound

8. OPTIMIZATION

In the course of SFE, for the enhancement of the extraction yield of the target compound, there are several parameters such as temperature, pressure, percentage of co-solvent, and sample size that need to be optimized, besides that solubility and mass transfer resistance is also associated with those parameters. Solubility is one of the main factors that can influence the effectiveness and the quality of the desired

product so we need to maximize the solubility of the extractable compound [28].

Optimization can be performed of the supercritical fluid extraction of vinblastine from *C. roseus* using ethanol as a co-solvent for CO_2 at different temperatures and pressures. Along with this, the co-solvent ratio can also influence the amount of extract. The static and dynamic extraction time can also be optimized to increase the yield. The yield of the solvent decides the fate of the extracted product purity, and the co-solvent ratio should be 2, 5, and 10% w/w. Among all the modifiers ethanol, methanol, acetonitrile, and n-hexane, the extraction yield with ethanol as a CO_2 modifier was higher, ethanol is used for extraction and micronization [27].

Micronization refers to the process of further reducing the size of extracted particles to a nanometer scale. It is done to increase efficiency. Application of micronization includes the production of active chemical ingredients, pharmaceuticals, food ingredients, etc.

8.1 Effects of Temperature and Pressure

Since the temperature and pressure can influence the solubility of a component during the extraction, hence they are of utmost importance when it comes to their influence on extraction efficiency. Increasing the pressure at specific temperatures increases the density of the solvent and solubility of the target compound [14]. Thus, the pressure is inversely proportional to the solvent volume needed for a particular extraction. This is not applicable to all the substances and target compounds because higher pressure can result in compacted raw material and can adversely affect the extraction yield.

8.2 Effects of Co-solvent/Modifier

An organic solvent may dissolve with the supercritical fluid when added at various proportions to CO_2 and can retain a considerable solvent power towards the targeted compounds [16]. CO_2 can only be used for the extraction of polar compounds. To increase the efficiency, polar co-solvent such as ethanol, methanol, water, acetic acid, and others are added to improve the solvation power of SC-CO_2. Methanol and ethanol are frequently used at concentrations below 10% of the quantity of CO_2 employed for the extraction [29]. Co-solvent affects the extraction yield as well as the bioactive properties of the extracts such as anti-inflammatory and antioxidants activities of extract.

8.3 Effect of Raw material

Many factors of the raw material can affect the extraction yield of the SFE such as moisture, porosity, particle size, and surface area. The sample to be extracted must be dried to lessen the moisture content, as the water content in the sample can compete with the extractable solute to associate with the solvent and lower the extraction yield [15]. In some cases, water may be helpful to permit the interaction of solvent and solute [16]. The mass transfer rate can be impacted by porosity and particle size. Reducing the particle size increases extraction efficiency because diminished particle size reduces the diffusion path of the solvent and increases the contact surface area which results in the acceleration of the extraction process.

8.4 Influence of extraction time

The extract's composition is affected by the extraction time. Both if extraction time is less or too long can decrease the yield and efficiency. If the time is short it can lead to incomplete extraction and if the time is too long it can result in solvent wastage. The extraction time is inversely proportional to the flow rate [25], [29].

9. ADVANCEMENTS

To improve the effectiveness of supercritical fluid extraction using carbon dioxide, many strategies which are eco-friendly have been implemented via enhancement of selectivity and yield. These strategies primarily include pre-treatment of biomass and modification of the solvent. The extraction condition depends on the target molecule and the properties of the plant material [18]. The addition of these extra processing steps improves the accessibility of the supercritical solvent to the solute by mechanistic disruption of the plant cell wall bringing about elevated extraction yield and efficacy [18]. Another approach is to treat the Catharanthus samples by using an enzymatic mixture before the extraction procedure. It has been reported that the use of fractionation operations during the extraction or separation improves selectivity. It is of extreme importance when there are several compounds that are required to be recovered from the plant as in the case of vinblastine extraction [30], [31].

10. CONCLUSION

Supercritical fluid extraction of *Catharanthus roseus* was done using ethanol as co-solvent and carbon dioxide at 300 bar at different temperatures. 2% of ethanol concentration which was used as co-solvent exhibited higher selectivity, at 10% condition indicated higher mass yield. When traditionally extracted using the solid-liquid method, the result presented a concentration up to 92.41% higher for Co_2+ethanol 2%, 300 bar, 40°C. On the other hand, VBL extraction using carbon dioxide and ethanol mixtures at high pressure could be used as an alternative to traditional phytochemical methods.

11. DISCUSSIONS

The extraction of vinblastine was performed within the specified temperature and pressure range, 35-60°C and 100-300 bar. Ethanol was not added as a co-solvent. Each step of the analysis purification process was done twice or thrice for accurate results. Extraction was done using pure carbon dioxide. The CO_2 flow rate was set to 300cc/min. The analysis of extraction was done by HPLC and MS analysis. The MS analysis is susceptible to temperature and pressure and therefore the temperature was set at 40°C and the pressure was 400 bar. Better sensitivity for vinblastine analysis could be achieved in MS using the positive ion mode in comparison with a UV detector (254 or 298 nm). Using this method, it was possible to detect levels as low as 1.00 ng of these alkaloids with standard samples [32]. MS analysis was operated in the positive ion mode in which scan range is kept up m/z = 100 to 1500. Concentrations of the standard solution used in this experiment were in the range of 0.4-30 mg/ml, and the linear correlation coefficient (g_2) of vinblastine was determined to be 0.9965. Results showed that the best vinblastine yield at 50°C was at CO_2 consumption 450g and pressure 400 bar. 100 bar pressure and 35°C temperature yield the highest concentration of vinblastine. At 70°C and 300 bar pressure, vinblastine yield increases exponentially when CO_2 consumption is beyond 400 bar [4]. Interstitial fluid velocity in the mixture is $4.70*10^{-4}$ m/s. Extraction bed length is 0.073 m and radius is 0.015 m. The porosity of the extraction bed used is 0.8907. The weighing factor is ½ [4]. At 50°C and 300 bar the vinblastine per unit gram of extract is 266.91 micrograms.

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