Chapter 4

SUPERCRITICAL FLUID EXTRACTION
4.1 Introduction

This chapter discusses the exploratory experiments conducted for optimization of extraction parameters using Supercritical CO₂ (SCF- CO₂). Optimization of extraction conditions in any SCFE experiment is important as it ensures maximum recovery of the analyte of interest. However, in natural product matrices, this is sometimes difficult to achieve unless the structures of the target analytes are known prior to extraction. The optimal conditions for conducting the extraction would best be predicted by considering the physical and chemical properties of the analyte of interest. The independent variables studied for optimization of extraction conditions were temperature, pressure, CO₂ flow rate and co-solvent (ethanol) percentage which are discussed in this chapter.

4.2 Experimental

4.2.1 Collection and authentication of plant material

Leaves of Nyctanthes arbor-tristis L. were collected locally. Identification of the plant species was confirmed at Agharkar Research Institute, Pune. The leaves were separated from the stalks, thoroughly washed with tap water and rinsed with distilled water. Leaves were dried at 40°C, powdered in order to increase the surface area of sample and passed through the sieve (mesh size=850µM) to maintain constant particle size throughout the study.

4.2.2 Chemicals and Reagents

Sodium carbonate (Na₂CO₃), Potassium acetate (CH₃COOK), Methanol, Ethyl acetate and Ammonia solution were of AR (analytical grade) purchased from Merck Pvt. Ltd. Aluminum chloride (AlCl₃) was purchased from Loba Chemie. Ethanol (95%) was purchased from Changshu Yangyuan Chemicals. Methanol used was of HPLC grade supplied by S.D. Fine Chem.Ltd., with minimum assay 99.8% by GLC. Standard
Quercetin of purity > 99.8% was purchased from Sigma Aldrich. Water used was distilled water which was freshly distilled whenever required. Carbon dioxide used was 99.9% pure obtained from Rakhangi. Before filling the gas in cylinder it was passed through a large bed of silica gel to remove the moisture in the gas.

4.2.3 Instrumentation

4.2.3.1 Supercritical Fluid Extraction (SCFE) System

For the present study, extraction was carried out in JASCO 900 series Supercritical fluid extractor using CO₂ in a supercritical state. The SCFE system and components were acquired from JASCO (Japan Spectroscopic Co.) 900 series. Figure 4.1 shows the Supercritical Fluid Extraction (SCFE) System used in the present study.

![Figure 4.1: Supercritical Fluid Extraction System](image)
The Supercritical fluid extractor included the following: 100 ml extraction vessel, temperature control unit (JASCO C0-965), high-pressure pump (JASCO-PU-980) and automated back pressure regulator (JASCO 880-81). The refrigerating coolant circulator was manufactured by Scinics Co. Ltd.

4.2.4 Other equipments and accessories

- Analytical Weighing Balance (Make and model: Shimadzu AUX 220). The range of balance was from 10mg to 200gm with a sensitivity of 0.1mg.
- UV spectrophotometer (Make and model: JASCO V 550 UV spectrophotometer). The software used data acquisition and evaluation was Spectra Manager.
- Ultrasonic bath (Make : Enterch electronics Pvt. Ltd )
- Speedovap (Make: Takahe Instruments)
- Vortex (Make : Spinx)
- Oven (Make: Expotitech)
- Grinder
- Refrigerator (Make: Samsung)
- Sieve of mesh size 850µ.
- Whatman Filter paper No.1
- Aluminium foil (Super wrap) used to cover the mobile phase.
- Ceramic wool used for packing of extraction vessel.
- Autopipettes used for transferring solutions were of variable range of 20-200µL, 100-1000µL and 1000-5000µL obtained from Eppendorf.
4.2.5 List of glassware’s

The following Class A calibrated glassware was used for different purpose during the study. All the glass apparatus were calibrated class A made up of inert glass material (Borosil, Riveria). Whenever light sensitive chemicals were to be handled amber coloured flasks and test tubes were used.

Volumetric flasks (10mL) used for standard stock solution and reagent preparation were made up of inert glass material (Borosil “Class A”). These flasks were available with a certification of calibration. Volumetric flasks (25mL, 100mL, 250mL) used to prepare solvents were made up of “Class B” type inert glass material.

Remaining glass wares used for different purposes during the experimental work were as follows:

- Glass pipettes: 10mL
- Glass beakers: 25mL, 50mL and 500mL
- Conical flasks: 100mL, 250mL and 500mL
- Measuring cycliners: 20mL, 100mL, 250mL
- Centrifuge tubes: 15mL
- Calibrated test tubes: 15mL

4.2.6 Preparation of Solutions

Stock solution of Quercetin
20mg Quercetin was dissolved in 80% ethanol and made upto 10mL volume to yield a concentration of 2mg/mL. This stock solution was further used to prepare standard calibration range according to each experimental requirement.
**Ethanol (80%; v/v)**
84.22mL of 95% ethanol was dissolved in distilled water and the volume was made upto 100mL to give 80% ethanol solution.

**Aluminum chloride AlCl₃ (10%; w/v)**
10g of AlCl₃ were dissolved in distilled water and the volume was made upto 100mL to give a 10% AlCl₃ solution.

**Potassium acetate CH₃COOK (1M)**
9.815g of CH₃COOK were dissolved in distilled water and the volume was made upto 100mL to give a 1M CH₃COOK solution.

**4.2.7 Packing of Extraction vessel**

The packing of the extraction vessel with plant material was done as follows. Firstly, the extraction vessel was rinsed and cleaned with acetone and was then dried using a stream of acetone. A small piece of ceramic wool was placed at the bottom of the vessel. Next, the powdered plant material (10g) was mixed well with 2.0mm diameter glass beads and placed in the extraction vessel. The introduction of some rigid materials such as glass beads with the ground sample have been shown to maintain a proper flow rate of CO₂ in the extractor vessel as well as they aid in obtaining the desired interaction between sample and solvent during the extraction process as reported (Chemat et al., 2004; Wang and Weller, 2006). Again a layer of ceramic wool was kept over the plant powder. The layers of ceramic wool prevented the physical carryover of the material during the passage of the supercritical fluid. The upper lid was then screwed tight and the vessel attached to the SCFE system.
4.2.8 Working of Supercritical Fluid Extractor

The SCFE apparatus used was a JASCO-Supercritical Fluid Extractor of the 900-series as described in Section 4.2.3.1. Figure 4.2 shows the schematic representation of working of SCFE system.

![Diagram of SCFE system](image)

**Figure 4.2: Schematic diagram of working of the SCFE system**

A fluid delivery system was used for delivering the fluid at a pressure in order to perform extraction. A two pump system consisting of reciprocating pumps was used for fluid delivery in SCFE as well as for the dynamic mixing of the co-solvent and the gas. The instrument incorporated an on-line addition facility to the supercritical fluid.
The flow rates of CO\(_2\) and co-solvent could be changed from 0.01 to 10.0 mL/min. Both pumps were operated at constant flow rate (CF) modes for CO\(_2\) and co solvent, all through the study. The CO\(_2\) gas first had to be cooled to -5.6°C before compression took place. This was done by passing the gas through the pre-cooling coil that was immersed in the coolant before introducing it to the pump. The refrigerating coolant circulator required a power of 230V AC 50/60 Hz, 10A. The maximum circulation capacity was 3.0 L/min. L.R. grade methanol was used as coolant. The dimensions of the cooling circulator were 17 x 38 x 52 (W x D x H) cm.

It should be noted that the fluid delivered by a cooled pump is not a supercritical fluid but a liquefied gas. To obtain supercritical fluid, the temperature of the liquefied gas was elevated to above the critical temperature of the fluid by using JASCO CO-965 series air circulating oven whose temperature could be maintained at any value between 35-80°C ± 1°C. The dimensions of the oven were 15 x 45 cm (W x H).

A preheating coil, present in the oven was employed for obtaining the supercritical fluid from the liquefied gas. A typical preheating coil is a coil of stainless steel tube with appropriate dimensions. The coil used was 0.5mm I.D x 1/16 in O.D. x 5-10m length, kept in the oven. It should be noted that the laminar flow in the coil causes a resistance of heat transfer. In order to reduce this resistance, the preheating tube must be coiled with a coil diameter as small as possible to evolve a turbulent flow in the tube.

The supercritical fluid CO\(_2\) and the co-solvent were mixed in the mixing vessel which was assembled in the oven. The vessel was a cylindrical vessel made up of SS 316 grade material. The length of the vessel was 15cm. The internal diameter of the vessel was 1cm and the wall of the vessel was 4mm thick. The volume of the vessel was 15ml. The vessel was provided with Teflon balls. The vessel was connected to the SCFE system using gas tight, 1/16”, stainless steel – 316 grade, ferrules at both the ends.
The extraction vessel used was a Stainless steel cylinder having the maximum operating pressure of 45 MPa and 50mL capacity. The lid was securely fastened to the vessel body by the knurled nut, and sealed with a high-durability PTFE O-ring. Packing of plant material in the extraction vessel was carried out as described in Section 4.2.7 and the supercritical carbon dioxide was introduced through the inlet tube.

The pressure of the liquefied gas also needs to be higher than the critical temperature of the fluid. If the pressure is lower than the critical temperature, evaporation takes place in the preheating tube and a large amount of energy is consumed as the evaporation energy. The desired pressure was achieved by using a JASCO 880-81 Back pressure regulator which worked in the range of 7.18 – 44.88 MPa. During the extraction, the flow resistance of the back-pressure regulator changes automatically to enable the system pressure to remain constant at the preset value. The pressure of the fluid was released by the back-pressure regulator and the fluid containing the extract flowed out from the outlet as an ordinary gas or a solvent, and it was collected.

All the extractions were carried out in the dynamic mode of SCFE, wherein the sample was constantly swept with fresh supercritical fluid at a flow rate determined by the extraction pressure and the dimensions of the outlet restrictor. Dynamic SCFE continually provides new fluid to the sample, and is more effective when the supercritical fluid is likely to become saturated with the target analytes.
4.2.9 Preliminary Extraction of flavonoids using SCFE method

A preliminary extraction of flavonoids using Supercritical carbon dioxide (SC-CO\(_2\)) was performed using 5gms of plant powder as starting extraction material. Extraction of flavonoids from the leaf powder of \(N.\) arbor-tristis was carried out by setting preliminary SCFE conditions as follows: temperature at 40°C, pressure at 19.61MPa, \(\text{CO}_2\) flow rate at 2.0mL/min and 9.09% co-solvent percentage, according to the literature discussed in the Section 2.4.8. Presence of flavonoids in the Supercritical Fluid (SCF) extract was checked by employing optimized Aluminium chloride (AlCl\(_3\)) method as described below.

4.2.10 Quantification of flavonoids by Aluminium chloride (AlCl\(_3\)) method

In the present study, quantification of flavonoids in all the SCF samples was carried out using Aluminium Chloride method as reported by Chang et al., (2002). Quercetin was used for standardization of the method.

**Principle**

The principle of the aluminum chloride colorimetric method is that aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, aluminum chloride forms acid labile complexes with the orthodihydroxyl groups in the A- or B-ring of flavonoids (Mabry et al., 1970). Plant extracts containing flavonoids react with aluminium chloride to develop a yellow colour which is read spectrophotometrically at 415nm.

**Procedure of AlCl\(_3\) method**

Standardization of AlCl\(_3\) method was carried out using three concentrations of Quercetin i.e. 25, 50 and 100 \(\mu\)g/mL as reported by Chang et al., (2002). From the Stock solution of
Quercetin (described in Section 4.2.6), 5mL was taken in 10mL volumetric flask and made upto 10mL volume using 80% ethanol to give working Quercetin solution of 1mg/mL. Aliquots of 0.25, 0.5 and 1mL from this 1mg/mL Quercetin working solution were taken separately in 3 different 10mL calibrated tubes and further diluted with 80% ethanol to get concentration range from 25, 50 and 100 µg/mL, respectively. Each standard solution (0.5 mL) was separately mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1M potassium acetate and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415nm with JASCO V-550 UV-Visible spectrophotometer. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in the blank. Similarly, 0.5 mL of preliminary SCF extract was reacted with aluminum chloride for determination of flavonoid content as described above. SCF extract was analyzed in triplicate. The total flavonoid content was expressed as quercetin equivalents (QE) in µg/g from a calibration curve of quercetin standard solution.

4.2.11 Method development of SCFE parameters

During optimization of SCFE conditions, each extraction parameter was optimized separately, one being tested (extraction pressure, extraction temperature, CO2 flow rate and co-solvent percentage) was varied keeping the other parameters constant. In the present study, a range of experiments were conducted for each parameter as described below. The parameters designed for optimization were according to available literature as discussed in Section 2.4.8. Dynamic extraction time was kept constant for 1h for the study of each parameter.

As discussed in literature review in Section 2.4.8, density of supercritical CO2 is altered depending on extraction temperature and pressure thereby affecting the extraction efficiency of SC-CO2. Density of CO2 decreases with increase in temperature at constant pressure while on the other hand, at constant temperature; increase in pressure values increases the CO2 density. Thus both the parameters affect the solvating power of CO2.
Therefore optimization of SCFE parameters was started by temperature and pressure study, followed by CO₂ flow rate and lastly co-solvent (ethanol) percentage.

**Parameter 1: Temperature study**

Effect of temperature on flavonoid extraction was studied in the range of 35°C to 70°C at an interval of 5°C, with constant pressure of 7.84MPa and CO₂ flow rate at 2.0mL/min and co-solvent percentage of 9.09%.

**Parameter 2: Pressure study**

Effect of pressure on flavonoid extraction was studied in the range of 7.84MPa to 29.41MPa by setting optimum temperature at 40°C and CO₂ flow rate at 2.0mL/min and co-solvent percentage of 9.09%.

**Parameter 3: CO₂ Flow Rate study**

The next step was to determine the optimal CO₂ flow rate for flavonoid extraction. The different flow rate values set were from 1.8mL/min to 3.5mL/min by keeping optimum temperature at 40°C and optimum pressure at 24.51MPa and constant co-solvent percentage of 9.09%.

**Parameter 4: Co-solvent percentage study**

The last parameter studied was the percentage of co-solvent for maximum extraction of flavonoids from the selected plant. As discussed in literature review (Section 2.4.8) polarity of neat carbon dioxide is changed by addition of co-solvent which improves extractability of SC-CO₂ for extraction of polar compounds. There was a need for the addition of small amounts of the polar co-solvent, to change the polarity of supercritical CO₂ for its use in flavonoid extraction. Murga *et al.*, (2000) have reported that when methanol and ethanol are used as co-solvents in their study; results obtained were qualitatively similar. In a study by Cavero *et al.*, (2006); they selected ethanol as a co-solvent and used different amount of ethanol for supercritical fluid extraction of oregano
leaves. Ethanol is reported to belong to the category of Generally Regarded As Safe Solvents (GRAS) Dunford, (2004). Therefore, taking into consideration the need for a co-solvent and lower toxicity of ethanol, effect of co-solvent percentage on extraction was studied by selecting ethanol at different percentages from 6.97% to 13.04% (v/v). Remaining SCFE conditions set were an optimum temperature of 40°C, optimum pressure of 25.41 MPa and with a flow rate of CO₂ at 2.0 mL/min.

For each of the parameters studied, CO₂ carrying the crude SCF extract flowed out of the extraction vessel unit and was collected in a collection tube. All the ethanolic SCF extracts were stored in amber coloured tubes in a refrigerator at 4°C until further analysis.

4.2.12 Quantification of flavonoids in SCF samples

Optimization of SCFE conditions for each parameter was carried out using optimized AlCl₃ method as described in Section 4.2.10. Quercetin was used as a standard to make the calibration curve. From the stock solution of Quercetin (as mentioned in Section 4.2.6), aliquots of 0.062, 0.125, 0.250, 0.500, 1.0, 2.0 and 3.0 mL were taken separately in 6 different 5 mL volumetric flask and further diluted with 80% ethanol to get concentration range from 25-1200 µg/mL. For estimation of Total Flavonoid Content (TFC), 0.5 mL of each SCF extract was used as the test solution and were reacted with aluminum chloride same as that for Quercetin. Remaining procedure followed was same as described in Section 4.2.10. All the samples were analyzed in triplicate. Total Flavonoid Content (TFC) for each tube was determined using a calibration curve of standard Quercetin and expressed as Quercetin equivalents (QE) in µg/g.

4.2.13 Qualitative phytochemical test for flavonoids

Presence of flavonoids in the crude SCF extract obtained under optimized conditions was checked by qualitative phytochemical test (Edeoga et al., 2005). A portion of crude SCF
extract (5mL) was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was then filtered through Whatman filter no.1. 1mL of dilute ammonia was mixed with 4mL of filtrate and observed for yellow coloration.

4.2.14 Scale up of SCF extraction

Using the results obtained from optimization studies and qualitative test, scale up of SCFE was undertaken by increasing the starting plant material to 1000gms. Scale up extraction was carried out by employing optimized SCFE conditions in order to obtain higher extraction yield of flavonoids.

4.2.15 Determination of Extraction yield

Ethanolic SCF extracts obtained under optimized SCFE conditions were collected and pooled together. The pooled extract was filtered through Whatman filter paper No. 1 to remove solid particles, if any. The filtered extract was then completely dried in the oven at 40°C and the final constant weight was recorded. Yield was calculated based on Equation 4.1 as shown below.

\[
Y_{\text{extract}} = \frac{m_{\text{extract}}}{m_{\text{herb}}} \times 100 \quad \text{[Equation 4.1]}
\]

Where \(Y_{\text{extract}}\) is the % extraction yield, \(m_{\text{extract}}\) is the crude extract mass (g) and \(m_{\text{herb}}\) is the extracted herb mass (g).
4.3 Results and Discussion

4.3.1 Preliminary analysis of SCF extract by AlCl₃ method

Initial flavonoid content in SCF extract obtained under preliminary SCFE conditions was determined using optimized AlCl₃ method. Table 4.1 shows the data obtained for Quercetin during standardization of AlCl₃ method. An important observation was noted while analyzing SCF extract by AlCl₃ method. Addition of AlCl₃ in SCF extract resulted in development of yellow colour indicating reaction of flavonoids present in sample with AlCl₃.

<table>
<thead>
<tr>
<th>Concentration of Quercetin (μg/mL)</th>
<th>Absorbance at 415nm (Mean ± S.D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0.1109 ± 0.008</td>
</tr>
<tr>
<td>50</td>
<td>0.2114 ± 0.019</td>
</tr>
<tr>
<td>100</td>
<td>0.3243 ± 0.026</td>
</tr>
<tr>
<td>150</td>
<td>0.4216 ± 0.0103</td>
</tr>
<tr>
<td>200</td>
<td>0.5380 ± 0.022</td>
</tr>
<tr>
<td>250</td>
<td>0.6407 ± 0.020</td>
</tr>
</tbody>
</table>

Table 4.1: Quercetin absorbance at 415nm using AlCl₃ method

Mean absorbance obtained for preliminary SCF extract at 415nm was 0.3289 ± 0.018 which indicated presence of flavonoid-AlCl₃ complex formation. However, further optimization of SCFE parameters was carried out to increase extraction efficiency of SC-CO₂.
4.3.2 Effect of Temperature on flavonoid extraction

Effect of different Temperature levels on flavonoid extraction was studied in the range of 35°C to 70°C. Figure 4.3 shows a linear calibration curve (I) of Quercetin, in the range of 25-1200μg/mL with co-efficient of determination ($R^2$) value of 0.998. The flavonoid content obtained for each temperature level is depicted in Table 4.2.

![Figure 4.3: Calibration curve (I) of Quercetin](image-url)
Table 4.2: Total Flavonoid Content (TFC) at different temperature levels

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Mean Absorbance</th>
<th>Total Flavonoid Content (µg QE/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>0.343</td>
<td>855 ±1.887</td>
</tr>
<tr>
<td>40</td>
<td>0.402</td>
<td>1002.5 ± 0.750</td>
</tr>
<tr>
<td>45</td>
<td>0.392</td>
<td>978.333 ± 0.511</td>
</tr>
<tr>
<td>50</td>
<td>0.345</td>
<td>861.583 ± 0.629</td>
</tr>
<tr>
<td>55</td>
<td>0.259</td>
<td>645 ± 0.721</td>
</tr>
<tr>
<td>60</td>
<td>0.165</td>
<td>408.583 ± 0.510</td>
</tr>
<tr>
<td>65</td>
<td>0.088</td>
<td>217.25 ± 0.520</td>
</tr>
<tr>
<td>70</td>
<td>0.074</td>
<td>184.416 ± 0.381</td>
</tr>
</tbody>
</table>

Key: Each value is expressed as Mean ± S.D (n=3)

Figure 4.4 presents the effect of different temperature levels on total flavonoid content of *Nyctanthes arbor-tristis* leaves. As observed in figure, an increase in flavonoid content was observed from 35°C to 40°C. This was, however, followed by a gradual decrease in flavonoid content as temperature was increased further. Near the critical pressure (Pc = 72.8 atm), the fluid density is very sensitive to temperature (Lang and Wai, 2001). This could possibly be the reason for extraction efficiency exhibiting a change at each temperature level.
As explained by Reverchon and De Marco, (2006), a rise in temperature causes an increase in the vapor pressure of the analytes which is greater than the reduction in the density of CO₂. Therefore the tendency of compounds to be extracted using through the supercritical fluid extraction technique also increases. Thus the initial increase in flavonoid extraction could be attributed to the improved vapor pressure of analytes. However, at constant pressure, increase in temperature causes a decrease in density of CO₂ thereby affecting the solvating power for SC-CO₂. Furthermore, Roop et al., (1989) reported that a moderate increase in temperature leads to a large decrease in fluid density thereby reducing solute solubility. Therefore this could be the possible reason for decrease in flavonoid extraction after 40°C. These observations are similar to those recoded earlier by Wang et al., (2008) and Salleh et al., (2009) in their studies on *Pueraria lobata* and *Strobilanthes crispus*, respectively.
4.3.3 Effect of Pressure on flavonoid extraction

Effect of different Pressure levels on flavonoid extraction was studied in the range of 7.84MPa to 29.41MPa. Figure 4.5 shows a linear calibration curve (II) of Quercetin, in the range of 25-1200μg/mL with a coefficient of determination (R²) value of 0.995 while Table 4.3 shows the flavonoid content obtained at each temperature level.

![Figure 4.5: Calibration curve (II) of Quercetin](image)
Table 4.3: Total Flavonoid Content (TFC) at different Pressure levels

<table>
<thead>
<tr>
<th>Pressure (MPa)</th>
<th>Mean absorbance</th>
<th>Total Flavonoid Content (µg QE/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.84</td>
<td>0.195</td>
<td>499.583 ± 0.763</td>
</tr>
<tr>
<td>9.8</td>
<td>0.214</td>
<td>545.5 ± 1.887</td>
</tr>
<tr>
<td>14.7</td>
<td>0.347</td>
<td>879.0 ± 1.520</td>
</tr>
<tr>
<td>19.61</td>
<td>0.380</td>
<td>960.916 ± 1.376</td>
</tr>
<tr>
<td>24.51</td>
<td>0.413</td>
<td>1044.167 ± 1.181</td>
</tr>
<tr>
<td>29.41</td>
<td>0.391</td>
<td>988.583 ± 1.376</td>
</tr>
</tbody>
</table>

Key: Each value is expressed as Mean ± S.D (n=3)

Figure 4.6 shows the effect of different pressure levels on flavonoid extraction from *N. arbor-tristis* leaves in SC-CO$_2$. As shown in the figure, there was increase in flavonoid content when pressure was increased from 7.84MPa to 24.51MPa. This could be due to an increase in density of SC-CO$_2$. At a constant temperature, density of the SC-CO$_2$ increases with rise in pressure thereby enhancing the solvent strength of SC-CO$_2$. As demonstrated by De Castro et al., (1999) and Salleh et al., (2009), the distance between the molecules decreases with increase in density; this enhances interaction between the analytes and CO$_2$, leading to greater solubility of the analytes in SC-CO$_2$. 
In addition, increase in pressure also accelerates mass transfer between the analytes and the solvent in a supercritical extractor vessel thereby improving the extraction process as explained by Thus the solubility of flavonoids in SC-CO$_2$ is directly proportional to the density of SC-CO$_2$. However, a further increase in pressure level above 24.51MPa resulted in a reduction in flavonoid extraction.

These observations are similar to the findings reported by Rezaei and Temelli, (2000). This could be attributed to the fact that a rise in pressure results in an increase in the fluid density, which in turn, alters solute solubility, thereby decreasing extraction (Gomes et al., 2001). These results coincide with those obtained earlier by Salleh et al., (2009) and Wang et al., (2008) in their research work.
4.3.4 Effect of CO$_2$ Flow rate on flavonoid extraction

Effect of different CO$_2$ flow rates on flavonoid extraction was studied in the range of 1.8mL/min to 3.5mL/min. Figure 4.7 shows a linear calibration curve (III) of Quercetin, in the range of 25-1200μg/mL with coefficient of determination ($R^2$) value of 0.996 and Table 4.4 shows the flavonoid content obtained at each temperature level.

![Figure 4.7: Calibration curve (III) of Quercetin](image.png)
Table 4.4: Total Flavonoid Content (TFC) at different CO$_2$ Flow Rate levels

<table>
<thead>
<tr>
<th>CO$_2$ Flow Rate (mL/min)</th>
<th>Mean Absorbance</th>
<th>Total Flavonoid Content (µg QE/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.8</td>
<td>0.408</td>
<td>97.9 ± 0.025</td>
</tr>
<tr>
<td>2.0</td>
<td>0.427</td>
<td>107.891 ± 0.425</td>
</tr>
<tr>
<td>2.5</td>
<td>0.416</td>
<td>99.175 ± 0.765</td>
</tr>
<tr>
<td>3.0</td>
<td>0.401</td>
<td>93.75 ± 0.132</td>
</tr>
<tr>
<td>3.5</td>
<td>0.348</td>
<td>89.25 ± 0.626</td>
</tr>
</tbody>
</table>

Key: Each value is expressed as Mean ± S.D (n=3)

In an attempt to investigate the effect of SC-CO$_2$ flow rate on extraction, a study was carried out in which CO$_2$ flow rate was varied from 2.0 to 3.5ml/min, keeping temperature and pressure constant. Figure 4.8 shows the effect of the solvent flow rate as an extraction parameter. As observed in the figure, flavonoid content increased with increase in flow rate, reached a maximum value and then decreased with increase in flow rate. A similar pattern was observed earlier by Kumoro and Hasan, (2007), in their study on *Andrographis paniculata*. The results obtained here can be explained as a trade-off between a mass transfer process and a thermodynamic equilibrium state as reported (Elkanzi and Singh, 2001). At low flow rates of the solvent, the mass transfer resistance limits the amount of solute transported into the bulk of the solvent and the supercritical carbon dioxide leaves the extractor unsaturated.
However, as reported by Mira et al., (1999) and Elkanzi and Singh, (2001) with an increase in flow rate, mass transfer resistance continues to decrease until the exiting solvent is saturated leading to an equilibrium being achieved and thereby resulting in maximum extraction. Furthermore, the excess amount of solvent that is not needed to penetrate the cellular structure of the leaves simply bypasses the extractable material as reported by Saldana et al., (2002). Thus for the present study, optimized CO₂ was 2.0mL/min.

4.3.5 Effect of Co-solvent (Ethanol) percentage on flavonoid extraction

The effect of different co-solvent percentages on flavonoid extraction was studied in the range of 6.97% to 13.04%. Figure 4.9 shows a linear calibration curve (IV) of Quercetin, in the range of 25-1200μg/mL with coefficient of determination (R²) value of 0.998 while Table 4.5 shows the flavonoid content obtained for each temperature level.
Figure 4.9: Calibration curve (IV) of Quercetin

Table 4.5: Total flavonoid content at different Co-solvent Percentage levels

<table>
<thead>
<tr>
<th>Co-solvent Percentage (%)</th>
<th>Mean Absorbance</th>
<th>Total Flavonoid Content (µg QE/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.97</td>
<td>0.405</td>
<td>1009.417 ± 1.941</td>
</tr>
<tr>
<td>8.25</td>
<td>0.432</td>
<td>1076.167 ± 1.040</td>
</tr>
<tr>
<td>9.09</td>
<td>0.428</td>
<td>1067.333 ± 0.877</td>
</tr>
<tr>
<td>9.9</td>
<td>0.415</td>
<td>999.312 ± 0.946</td>
</tr>
<tr>
<td>10.71</td>
<td>0.401</td>
<td>994.829 ± 0.877</td>
</tr>
<tr>
<td>11.5</td>
<td>0.399</td>
<td>972.083 ± 1.010</td>
</tr>
<tr>
<td>12.28</td>
<td>0.390</td>
<td>931.667 ± 0.520</td>
</tr>
<tr>
<td>13.04</td>
<td>0.374</td>
<td>18.323 ± 1.464</td>
</tr>
</tbody>
</table>

Key: Each value is expressed as Mean ± S.D (n=3)
Figure 4.10 shows the effect of co-solvent percentage on flavonoid extraction. This range of co-solvent was used basically to change the polarity of SC-CO$_2$. The co-solvent basically interacts with the analyte complex, causing a rapid desorption in SC-CO$_2$ thereby enhancing the solubility of the fluid as reported by Castro et al., (1996). As observed in the figure below, increase in flavonoid extraction was obtained only till 8.25% with further drop in flavonoid content.

![Figure 4.10: Effect of Co-solvent Percentage on Flavonoid Extraction](image)

Similar results were observed in another study conducted by Wang et al., (2008) in an attempt to understand the effect of co-solvent flow rate on the flavonoid yield from *Pueraria lobata*. Later in a study undertaken on spearmint leaves by Bimakr et al., (2010), a similar pattern was observed. Various percentages of ethanol used exhibited different effects in changing the fluid polarity and thus resulted in diverse effects on the solubility enhancement of the flavonoids.
The optimal extraction yield may be fulfilled when the polarity of the fluid and its flavonoids are coincident. In the study, decrease in flavonoid content was observed beyond a co-solvent concentration of 8.25%. This could be ascribed to the addition of large amounts of co-solvent. This would lead to a considerable change in the critical parameters of the mixture thereby changing the state of the fluids as observed by Tong and Imagawa, (1995). Thus the optimized co-solvent percentage for present study was 8.25%.

Thus on the basis of studies on extraction of flavonoids from *N. arbor-tristis*, the optimized parameters are shown in Table 4.6.

Table 4.6: Final Optimized SCFE conditions for flavonoids from *Nyctanthes arbor-tristis* leaves

<table>
<thead>
<tr>
<th>Operating Parameters</th>
<th>Optimized SCFE Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>40°C</td>
</tr>
<tr>
<td>Pressure</td>
<td>24.51MPa</td>
</tr>
<tr>
<td>CO₂ Flow Rate</td>
<td>2.0mL/min</td>
</tr>
<tr>
<td>Co-solvent Percentage</td>
<td>8.25%</td>
</tr>
</tbody>
</table>

4.3.6 Qualitative test for flavonoids

Crude SCF extract was qualitatively checked for presence of flavonoids by performing the ammonia test. Figure 4.11 shows the development of a yellow color at the bottom of the SCFE extract (indicated by arrow).
The development of a yellow color on addition of ammonia indicated the presence of flavonoids in the crude SCF extract indicating successful extraction of flavonoids from plant material under optimized SCFE.

4.3.7 Extraction yield

Under the optimized SCFE conditions, extraction yield was calculated using equation 4.1 as described in Section 4.2.15 and was found to be 69.85%
4.4 Conclusion

The important conclusions from this chapter are as follows:

- Till date, there are no reports of the use of Supercritical CO$_2$ as a solvent for the extraction of flavonoids from *N. arbor-tristis*. Hence this study could be regarded as the first attempt to optimize the extraction parameters for the extraction of flavonoid components from leaves of *N. arbor-tristis* employing this technique.

- The best optimized SCFE conditions for *N. arbor-tristis* leaves are temperature (40°C), pressure (24.51MPa), CO$_2$ flow rate (2.0mL/min) and co-solvent percentage (8.25%).

- In this study, different process parameters were observed to play significant role in changing the extraction efficiency by changing properties of supercritical fluid. Thus each parameter has a key role by influencing the extraction process.

- This study shows that Supercritical CO$_2$ extraction method can serve as an alternative for extraction of flavonoids.