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SUPERCRITICAL CARBON DIOXIDE (SC-CO_2) EXTRACTION OF BIOACTIVE COMPOUNDS FROM MULBERRY (*MORUS ALBA* L) LEAVES: PROCESS OPTIMIZATION

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ABSTRACT

A simple, more efficient, less time consuming extraction technique has been developed for extraction of rutin, a flavonoid and total phenolics from dry leaf powder of *M. alba* using supercritical carbon dioxide (SC-CO_2) equipment. Extraction parameters such as temperature (40, 50 and 60 °C), pressure (100, 150 and 200 bar) and dynamic extraction time (40, 60 and 80 min) were optimized using a factorial design (3^3). The best conditions obtained for SC-CO_2 extraction of flavonoids and phenolics were found within the experimental range of variables at a temperature of 50 °C, pressure of 200 bar and dynamic extraction time of 80 min. High performance liquid chromatography (HPLC) was used to determine the activity of the rutin from mulberry leaf powder extract. Apart from the optimum SFE condition, the other extraction condition at minimum (100 bar, 40 °C and 40 min) and maximum (200 bar, 60 °C and 80 min) level of each studied parameters were selected and analyzed by spectrophotometer.

Keywords: Flavonoids; *M. alba*; Phenolics; Rutin; Spectrophotometer and *Supercritical* carbon dioxide

INTRODUCTION

Mulberry (*Morus alba* L.) plant, belongs to the family "*Moraceae*" with genus "*Morus*" and species "*alba*". It is grown under varied climatic conditions ranging from temperate to tropical, and its leaves are mainly used to feed silkworms (*Bombyx mori* L.). Rutin, quercitin and other flavonoids have also been found in mulberry leaves. Recent studies indicated that mulberry leaf extracts could significantly reduce blood glucose, high blood pressure, high cholesterol, neutral fat and prevent thrombus formation and ageing. Mulberry leaf is one of the plants that contain the highest, 1-3 per cent of flavonoids in dried leaves (Chen *et al*., 2008).

Flavonoids and phenolics are plant-derived bio compounds known as natural antioxidants due to their redox properties, allowing them to act as free radical scavengers, hydrogen donors, reducing agents and metal ion chelators. Natural antioxidants have received considerable attention due to their ability to prevent human body against oxidative stress induced by imbalance between generation and removal of reactive oxygen species and retard the progress of many chronic diseases (Wang *et al*., 2011). Rutin is the only flavone, which has a clinical use and its main biological property is to antagonize the increase of capillary fragility, associated with a hemorrhagic disease and is used to treat capillary bleeding. The hypoglycemic activity of rutin has also been reported and is thus one of the active constituents in the leaves of *Morus alba* L., which also show the hypoglycemic effect (Dighe *et al*., 2011).

Conventional extraction methods, such as steam distillation and organic solvent extraction, have been used to extract bioactive compounds from plant materials for a long time. These methods usually require a long time, a large amount of solvent and high temperatures (Liza *et al*., 2010). Therefore, developing alternative extraction techniques with high efficiency and moderate peculiarity is highly desirable. Supercritical carbon dioxide (SC-CO2) extraction has received a great deal of attention because it is usually performed at low temperatures, costing short extraction time and a small amount of solvent (Li *et al*., 2010). Generally, addition of a small amount of a liquid polar modifier (methanol or ethanol) can significantly enhance extraction efficiency of flavonoids and phenolics (Lang and Wai, 2001).

To date, there are no publications found on supercritical fluid extraction of bioactive compounds including flavonoids and phenolics from mulberry (*Morus alba* L.) leaves**.** The aim of this study was to develop an optimal extraction condition using factorial design (3^3), to determine the total flavonoids and phenolics content in the extract using spectrophotometer and to identify the main flavonoid (rutin) using high performance liquid chromatography (HPLC).

MATERIALS AND METHODS

MATERIALS

The leaves of *Morus alba* were procured from

the mulberry garden of Sericulture section, College of Agriculture, Raichur, Karnataka, India. The leaves were separated from the stalk, thoroughly washed with tap water. The leaves were dried at room temperature (28±2 °C) until a constant weight was reached and then ground into powder manually. The powder was sieved using IS 500 µm sieve to get a fine dust.

Standards of rutin and gallic acid, HPLC grade water, acetic acid, acetonitril, Folin-Ciocalteu's phenol reagent, ethanol and other chemicals were procured from Sd Fine-Chem ltd., Bangalore.

SC-CO_2 EXTRACTION

The supercritical carbon dioxide extraction system (Make: Waters Thar;Model: SFE 500) used for extraction included the accessories like 500 ml extraction vessel, high-pressure pump, automated back pressure regulator, water bath and pump unit. Circulated deionized water (at 5 °C) was used for cooling different zones in the SC-CO_2 extraction system. 100 g of ground plant material (mulberry leaf powder) was placed into the 500 ml extraction vessel. The flow rate of CO_2 and modifier (ethanol) were maintained at 25 g/min and 3 g/min, respectively. Liquid CO_2 and modifier were pumped into the extraction vessel after desired temperature was achieved. In this study, three factorial design was applied for optimization of process parameters. Extractions were performed at three levels of pressure (100, 150 and 200 bar), temperature (40, 50 and 60 °C) and dynamic extraction time (40, 60 and 80 min). The extracts were collected in a glass vial at room temperature and atmospheric pressure. The modifier was removed completely by a vacuum rotary evaporator (Make: Superfit, Rotavap; Model: PBU-6D) at 40 °C (water-bath temperature). The dry extracts were adjusted to 50 ml with absolute ethanol as samples for further analysis.

TOTAL FLAVONOID CONTENT (TFC)

TFC of the extract was measured using the method described by Nie *et al*. (2012). To one ml of the concentrated extracted solution, about 0.5 ml of 5 per cent $NaNO_2$ was added, shaken for 5 min, and further about 0.5 ml of 10 per cent Al $(NO_3)_3$ was also added and shaken for 5 min. 4 ml of distilled water and 4 ml of 4 per cent NaOH were then added, and the tubes were shaken for 15 min and the absorbance was determined using spectrophotometer at 510 nm. Rutin was used as an equivalent standard. Standard curve of rutin was used to estimate the concentration of flavonoids. The analysis was performed in triplicate.

TOTAL PHENOLIC CONTENT (TPC)

Total phenolic content of the extract was determined by following using the Folin–Ciocalteau reagent (FCR) method (AOAC, 2005). Two ml of saturated lead acetate solution was added drop wise to 5 ml of supercritical fluid extract and saturated solution of disodium hydrogen phosphate was added drop wise till the precipitation was complete. Solution was mixed thoroughly and kept it for overnight for colour clarification of the extract. Extract was then filtered through Whatman No. 41 filter paper and made upto a known volume of 25 ml with ethanol. Clarified solution (0.2 ml) was taken in a test tube and made volume up to 1ml with distilled water, to that 1 ml of freshly diluted Folin–Ciocalteau reagent was added. 2 ml of sodium carbonate (2 g) dissolved in 100 ml of 0.1N NaOH solution was added to the mixture and mixed throughly. The absorbance was measured at650 nm against a blank of distilled water using a spectrophotometer (Make: Systronics; Model: PC based double beam spectrophotometer 2202). Gallic acid was used as an equivalent standard. Standard curve of gallic acid was used to estimate concentration of phenols. The analysis was replicated thrice.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ANALYSIS

SAMPLE PREPARATION

The sample was filtered through a filter paper and centrifuged at 5000 rpm for 10 min, then filtered again through disposable 0.22 µm nylon syringe filters and kept ready to inject in HPLC (Qun, 2011).

b) Standard preparation

A rutin standard (0.011 g) was accurately weighed and dissolved in 10 ml of ethanol. From this 5 ml was drawn and made up to known volume of 25 ml with ethanol. About 1, 2, 4, 6, and 8 ml of rutin standard solution were diluted to 10 ml by ethanol. Using the concentration of the rutin standard solution as the abscissa and the peak area as the*y*-coordinate, the linear chart was constructed (Nie *et al*., 2012).

The UV detection system was connected to a reversed-phase column (Shimpack-XR-ODS-III 150×3.0mm, 2.1µm). The HPLC mobile phase included solvent A - acetonitril and solvent B - 0.5 per cent acetic acid (15:85, v/v). Absorbance was measured at 350 nm for the rutin detection. The flow rate was 0.5 ml/min, and the column temperature was maintained at 30 °C. The sample acquired from the optimal system was injected with a volume of 10 µl after dilution. The peak area was used for quantification of the compound (Qun, 2011).

STATISTICAL ANALYSIS

The experiments were conducted with factorial design (3^3), which refers to three independent variables at three levels each were conducted for each dependent variable. Altogether, 27 runs were performed under randomized order and the experiments on each sample were carried out to evaluate the variability of measurement. The results were evaluated by analysis of variance (ANOVA). These analyses were performed using the software, Design Expert - Version 7.7.0 (State-Ease, Minneapolis, MN).

RESULTS AND DISCUSSION

OPTIMIZATION OF SC-CO_2 EXTRACTION PROCESS

The experiment was conducted to optimize SFE process parameters at a CO_2 flow rate of 25 g/min and a modifier (ethanol) flow rate of 3 g/min and the results are presented in Table 1. The results showed that the

maximum total flavonoid content (TFC) and total phenolic content (TPC) of the extracts were 34.87 mg/g dry material and 27.82 mg/g dry material, respectively. ANOVA (Table 2(a) and Table 2(b)) showed that all the process parameters had a significant ($p < 0.01$) effect on both TFC and TPC of the extracts. The best conditions obtained for SC-CO_2 extraction of flavonoids and phenolics from mulberry (*M. alba*) leaves was 200 bar, 50 °C and 80 min. Optimized parameters had desirability of 0.99 depicting efficient extraction of desired product.

EFFECT OF SOLVENT TEMPERATURE AND PRESSURE ON TFC AND TPC IN MULBERRY LEAF POWDER EXTRACT

The effects of solvent temperature and pressure on total flavonoids and phenols from mulberry (*M. alba)* leaf powder extract at three levels of temperatures (40, 50 and 60 °C) and pressures (100, 150 and 200 bar) at constant dynamic extraction time are presented in Fig. 1. It is evident from the figure that, the TFC and TPC was increased with the increase in pressure from 100 to 200 bar. This might be due to increased SC-CO_2 density which caused the increase in solvent strength and solubility of the analytes in CO_2. It is also observed from the figure that the TFC and TPC were increased as temperature was increased from 40 to 50 °C. However, a temperature increase from 50 to 60 °C caused a decrease in the flavonoid and phenolic content which might be due to decreased fluid density and thus reduced the total flavonoids and phenols in the extract. The effect of pressure and temperature on extraction process was similar with the results obtained by Liza *et al*. (2010); Cao *et al*. (2007). However, both pressure and temperature had great influence on the recovery of antioxidant compounds. An increase in temperature resulted in a decrease in antioxidant compound recovery. This might be due to the fact that most of these antioxidant compounds, such as vitamin C, polyphenols and anthocyanins, are unstable and highly susceptible to thermal degradation (Cavalcanti *et al*., 2011).

EFFECT OF SOLVENT TEMPERATURE AND TIME ON TFC AND TPC IN MULBERRY LEAF POWDER EXTRACT

The effect of solvent temperature and time on SC-CO_2 extraction of total flavonoids and total phenols from mulberry (*M. alba)* leaf powder extract at three levels of temperatures (40, 50 and 60 °C) and dynamic extraction times (40, 60 and 80 min) at constant pressure are presented in Fig. 2. It is clear from the figure that the TFC and TPC increased as temperature was increased from 40 to 50 °C. However, a temperature increase from 50 to 60 °C caused a decrease in the flavonoid and phenolic yield which was probably due to reduction in the density of CO_2. A moderate increase in temperature can lead to a large decrease in fluid density, with a consequent reduction in solute solubility (Roopa *et al*., 1989). It is also visualized from the figure that by increasing the dynamic extraction time, the flavonoid and phenolic content was increased. The highest extraction time of 80 min is a reasonable time to be used for the extraction which contributes higher flavonoid yield. Similar effect of temperature and time was observed on extraction of bioactive flavonoids from *Strobilanthes crispus* (Liza *et al*., 2010).

Table 1- Effect of process variables on SC-CO_2 extraction of total flavonoids, phenols and rutin from mulberry leaf powder extract

Treatment	Pressure (bar)	Temperature (°C)	Time (min)	Total Flavonoids (mg/g)	Total Phenols (mg/g)	Rutin (mg/g)
T_o	Control			28.72	23.91	2.03
T_1	100	40	40	7.05	9.04	0.71
T_2			60	8.26	10.51	0.74
T_3			80	9.27	10.72	1.12
T_4	100	50	40	9.74	11.55	1.26
T_5			60	10.51	11.95	1.28
T_6			80	11.50	12.71	1.32
T_7	100	60	40	9.79	10.41	0.35
T_8			60	10.14	10.69	0.54
T_9			80	11.06	10.91	0.93
T_{10}	150	40	40	14.82	12.85	1.22
T_{11}			60	15.09	13.31	1.35
T_{12}			80	15.96	13.60	1.43
T_{13}	150	50	40	16.04	14.66	1.61
T_{14}			60	17.77	15.88	1.78
T_{15}			80	18.75	16.90	1.89

T_{16}	150	60	40	15.50	14.15	1.03
T_{17}			60	16.72	14.91	1.23
T_{18}			80	18.02	15.56	1.55
T_{19}	200	40	40	24.07	18.06	2.11
T_{20}			60	26.85	22.12	2.29
T_{21}			80	28.75	23.76	2.50
T_{22}	200	50	40	30.23	24.48	2.78
T_{23}			60	31.93	25.79	3.05
T_{24}			80	34.87	27.82	3.35
T_{25}	200	60	40	19.70	19.81	1.55
T_{26}			60	20.92	20.14	2.04
T_{27}			80	21.65	20.85	2.45

Table 2(a). ANOVA for interaction effect of temperature, pressure and time on extraction of total phenols from mulberry leaf powder

Source	Sum of Squares	DF	Mean Sum of Square	F Value	p-value Prob > F	SE±	CD @ 1 %
Model	1467.59	26	56.45	382.19	< 0.0001	significant	
A-Temp	98.36	2	49.18	333.01	< 0.0001	0.07	0.20
B-Pressure	1264.26	2	632.13	4280.11	< 0.0001	0.07	0.18
C-Time	35.42	2	17.71	119.90	< 0.0001	0.07	0.18
AB	45.30	4	11.32	76.68	< 0.0001	0.10	0.29
AC	6.67	4	1.67	11.29	< 0.0001	0.10	0.29
BC	8.93	4	2.23	15.11	< 0.0001	0.10	0.29
ABC	8.65	8	1.08	7.32	< 0.0001	0.15	0.41
Pure Error	3.99	27	0.15				
Cor Total	1471.57	53					
SD = 0.38; Mean = 16.02; R^2 = 0.99							

DF:Degrees of Freedom; SE: Standard Error; CD: Coefficient of Deviation; SD: Standard Deviation

Table 2(b). ANOVA for interaction effect of temperature, pressure and time on extraction of total flavonoids from mulberry leaf powder

Source	Sum of Squares	DF	Mean Sum of Square	F Value	p-value Prob > F	SE±	CD @ 1 %
Model	3097.17	26	119.12	2959.23	< 0.0001	significant	
A-Temp	181.42	2	90.71	2253.36	< 0.0001	0.04	0.11
B-Pressure	2585.21	2	1292.60	32110.96	< 0.0001	0.04	0.11
C-Time	58.21	2	29.10	723.00	< 0.0001	0.04	0.11
AB	257.44	4	64.36	1598.83	< 0.0001	0.05	0.15
AC	2.04	4	0.51	12.68	< 0.0001	0.05	0.15
BC	6.85	4	1.71	42.53	< 0.0001	0.05	0.15
ABC	6.01	8	0.75	18.65	< 0.0001	0.08	0.21
Pure Error	1.09	27	0.04				
Cor Total	3098.26	53					
SD = 0.20; Mean = 17.59; R^2 = 0.99							

DF: Degrees of Freedom; SE: Standard Error; CD: Coefficient of Deviation; SD: Standard Deviation

EFFECT OF SOLVENT PRESSURE AND TIME ON TFC AND TPC IN MULBERRY LEAF POWDER EXTRACT

The effect of solvent pressure and time on SC-CO_2 extraction of total flavonoids and total phenols from mulberry (*M. alba)* leaf powder extract at three levels of pressures (100, 150 and 200 bar) and dynamic extraction times (40, 60 and 80 min) at constant temperature are presented in Fig. 3. It is visualized from the figure that the flavonoid and phenolic yield was increased with increasing pressure from 100 to 200 bar to a certain value. This might be due to the increase in pressure which increased the density of SC-CO_2 thereby increased the solvent strength and solubility of analytes in CO_2. Over this range of pressure, increasing fluid density was presumably the main mechanism leading to a higher flavonoid and phenolic yield. It is also evident from the figure that by increasing the dynamic extraction time, the extraction yield enhanced. The 80 min is a reasonable time to be used for the extraction which contributes higher extraction yield. The effect of pressure and time in the present investigation was similar with the results obtained by Liza *et al*. (2010) for extraction of bioactive flavonoids from *Strobilanthes crispus.*

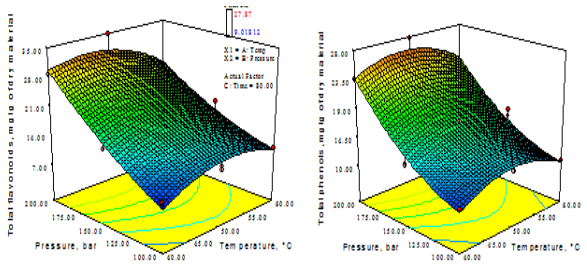

Fig. 1. Effect of extraction temperature and pressure on total flavonoids and phenols in mulberry leaf powder extract

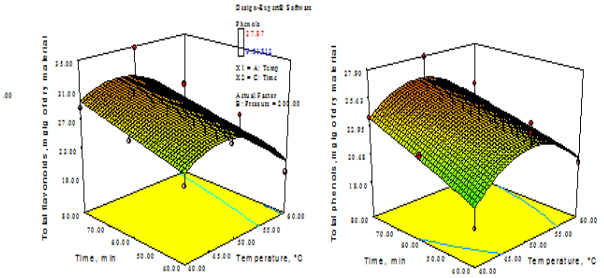

Fig. 2. Effect of extraction temperature and time on total flavonoids and phenols in mulberry leaf powder extract

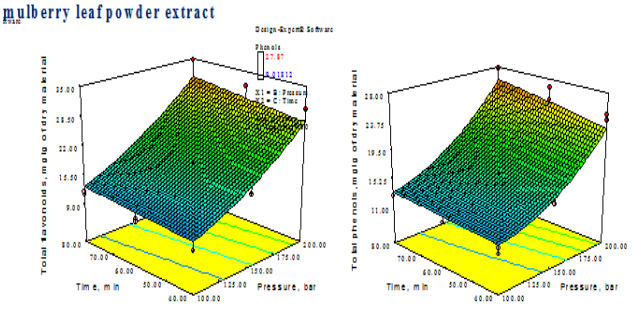

Fig. 3. Effect of extraction pressure and time on total flavonoids and phenols in mulberry leaf powder extract

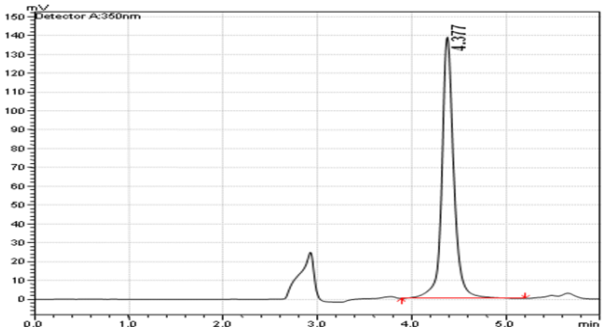

Fig. 4. HPLC Chromatogram for rutin standard (40 ppm)

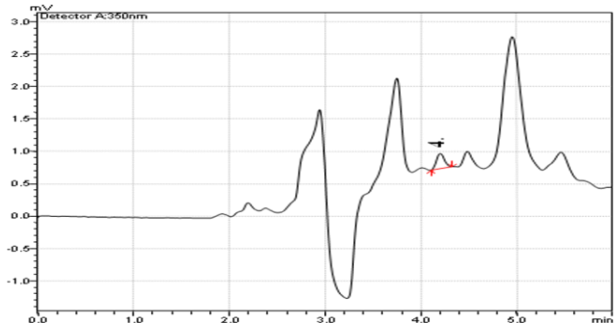

Fig. 5. HPLC Chromatogram for SC-CO_2 extraction of rutin at 40 °C, 100 bar and 40 min

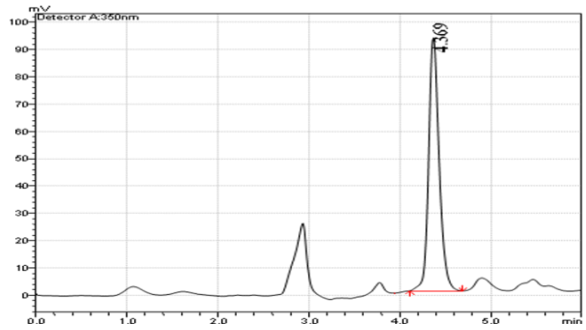

Fig. 6. HPLC Chromatogram for SC-CO_2 extraction of rutin at 50 °C, 200 bar and 80 min

QUANTIFICATION OF THE MAIN FLAVONOID (RUTIN) FROM SC-CO_2 EXTRACT

The rutin content in supercritical fluid extract of mulberry leaf powder obtained for different temperature, pressure and time combinations are presented in Table 1. The increase in SC-CO_2 pressure from 100 to 200 bar, temperature from 40 to 50 °C and time from 40 to 80 min there is a significant increase of rutin. However, a temperature increase from 50 to 60 °C there is a decrease in rutin content. The HPLC chromatogram of rutin standard is given in Fig. 4. Among all the treatment combinations, highest rutin content of 3.35 mg/g of dry material was recorded at SC-CO_2 pressure of 200 bar, temperature of 50 °C and dynamic extraction time of 80 min, which is considered as the optimum and best SC-CO_2 extraction condition for obtaining the highest rutin from mulberry leaf powder. The HPLC chromatogram for rutin at optimised condition is given in Fig. 6. The lowest rutin content of 0.29 mg/g

of dry material was observed at SC-CO_2 pressure of 100 bar, temperature of 40 °C and dynamic extraction time of 40 min. The HPLC chromatogram for rutin at minimum condition is given in Fig. 5. Radojkovic *et al*. (2012) have reported that the main flavonol glycoside of *M. alba* was rutin (2.89 mg/g). The variation of rutin in the extract depends on many factors, such as degree of maturity at harvest, genetic differences and environmental conditions.

CONCLUSION

Based on the results obtained, the optimal SC-CO_2 extraction condition to obtain highest extraction yield of flavonoids and phenolic content from mulberry leaves was found to be SC-CO_2 temperature of 50 °C, pressure of 200 bar and dynamic extraction time of 80 min. Under the optimum condition the highest rutin content of 3.35 mg/g of dry material was extracted. The identification of rutin by HPLC in this study clearly revealed that temperature of 50 °C is more convenient for SC-CO_2 extraction and to avoid thermal degradation of the sample. These results indicate that SC-CO_2 could be a promising alternative for preparation of extracts enriched with bioactive compounds from mulberry (*M. alba*) leaves.

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