RESEARCH ARTICLE

Supercritical CO_2 Extraction, Quantification and Pharmacological Screening of Steroidal Saponins from Fruits of *Momordica charantia* L

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ABSTRACT

Momordica charantia L. (MC), a Cucurbitaceae family member, is the most recognized plant for its hypoglycemic activity. Charantin, a steroidal saponin, is the most studied potent phytochemical in MC for diabetes. This research paper aims at the extraction, quantification and pharmacological screening of charantin, from fruits of MC. Extraction was performed by using traditional soxhlet extraction and modified supercritical fluid extraction (SFE) techniques, and compared the results of both techniques in terms of percent extract yield, quantity of charantin and *in-vitro* antidiabetic activity of both extracts. (Soxhlet extract and SC-CO_2 extract). Further, quantitative estimation of charantin in both extracts was done by HPLC-UV method and it was validated as per the ICH guidelines. When compared to soxhlet extract, the SC-CO_2 extract displayed high antihyperglycemic activity by blocking *α-amylase and* α-glucosidase enzymes. Study also indicates that SC-CO_2 extract had higher antioxidant activity (0.25 mg/mL) than soxhlet extract (0.33 mg/mL), signifying the SFE technique's efficiency over the traditional soxhlet extraction method. *In-vitro* antidiabetic study indicated that the biomolecule charantin extracted from fruits of MC possess potent antidiabetic and high antioxidant activities and, therefore, hold potential for manufacturing innovative natural remedies to treat diabetes and its complications with no side effects.

Keywords: Antidiabetic, Antioxidants, Bitter gourd, Charantin, HPLC-UV, *Momordica charantia*, Supercritical fluid extraction

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Conflict of interest: None

INTRODUCTION

Herbal remedies have been widely used around the world since antiquity. Around the world, phytotherapy has been used successfully for many years to treat various disorders using phytocompounds derived from plants.[1,2] In the early years, MC from the Cucurbitaceae family, sometimes known as bitter gourd locally, was considered a helpful dietary adjunct for diabetes[3] Various phytoconstituents are isolated from MC, like momordicin, momordin, charantin, charine, cucurbitins, diosgenin, momordicosides, stigmasterol, vicine, etc., which have a variety of medicinal properties like hyperlipidemic, antioxidant, antifungal, antidiabetic, anticancer and antitumor, anti-helminthic, antimicrobial, anti-inflammatory, and wound healing.[4,5] In this study, we focused on major component charantin, which is present in all sections of the plant but is particularly abundant in fruits. It is a steroidal saponin, also known as momocharin, with an insulin-like structure and properties. It is most studied potent phytochemical in a bitter gourd for diabetes and it is made up of 1:1 combination of two glucosides as, stigmasterol glucoside and *β*-sitosterol glucoside.[6]

It is a special molecule made up of a glucoside tail and a sterol head. Aglycone, the steroidal component, is very soluble in non-polar solvents and glucosides connected to its molecules is mildly soluble in polar organic solvents.[7] Therefore, a combination of solvents and a successful extraction technique are required for an efficient extraction of charantin to extract the non-polar and polar part simultaneously with good extract yield and charantin content. Bitter gourd has previously been extracted using a variety of traditional methods, including heating, boiling, maceration, refluxing, and soxhlet extraction, using common solvents, including ethanol, water, and methanol.[8]

Conventional extraction techniques require several hours or even days to complete, require a significant quantity of organic solvents, and require downstream processes to remove the toxic solvents. Bioactive chemical loss occurs due to the toxic and hazardous organic solvents, high temperatures,

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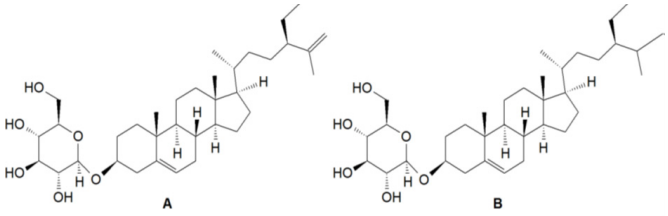

A: Stigmasterol glucoside B: β-sitosterol glucoside

oxidation and hydrolysis during extraction. In addition, this method's extract contains less bioactive compound.[9,10]

An effective method to extract different bioactive compounds from plants is SC-CO_2 extraction. It is a quick, environmentally safe, and effective technique for removing non-polar chemicals from plant matrices. Numerous benefits include a faster extraction rate, less solvent use, lack of organic solvents, and simplicity in separating the extract from the solvent.[11] SFE of flavonoids has been successfully carried out with high quantity of flavonoids.[12,13] Since the process operates at low critical temperatures and pressures, which prevent bioactive chemicals from degrading, it offers large quantity of bioactive compounds in the extract.

Because of its low cost, inert, non-flammable, non-toxic, naturally available in abundance low critical temperatures and pressure CO_2 gas is a popular solvent in SFE method. The Food and Drug Administration (FDA) also approved CO_2 solvent as safe. To produce the highest yield of the desired component, the solubility of supercritical CO_2 can be modified based on temperature and pressure.[14]

SC-CO_2 is limited in its ability to extract polar molecules due to its non-polar nature. To address this issue, polar modifiers are employed to extract polar compounds.[15] In the present work ethyl alcohol is utilized as a co-extractant due to its great miscibility with CO_2 and ability to extract both polar and non-polar molecules.[16,17] Nowadays, basic requirement in herbal drug development is the qualitative analysis of crude extracts.[18] For the study of bioactive molecules several extraction and analytical Techniques have been established. [19]

In the present investigation, HPLC-UV technique was used for the quantification of charantin in crude extracts as it is an easy, quick, exact and selective technique. Here, we compared conventional soxhlet and modern SC-CO_2 extraction techniques regarding yield of extract, percentage of charantin and antidiabetic activities of both extracts.

Diabetes mellitus (DM) is a major metabolic disease indicated by an abnormally high blood glucose level. It is a major global health issue.[20] Many synthetic antihyperglycemic drugs possess various side effects, including diarrhea, weakness, headache, nausea, kidney problems, and low glucose levels in the blood.[21-23] A significant barrier is medication resistance; sulfonylureas shows ineffectiveness following six years of therapy in 47% of diabetic people. Diabetes has become a major worldwide health problem due to a lack of therapeutic choices. The search for effective diabetes treatments with minimal or no side effects continues.[24]

Natural components or phytocompounds has long been used effectively to treat several diseases and disorders all over the world. In India several thousands of plant species are said to have medicinal properties.[25,26] Herbal substances or their derivatives account for over 50% of the pharmaceuticals that the FDA has accepted.[27] Extraction and estimation of biomolecules in exact from plants plays very crucial part in phytotherapy.

It is essential to extract bioactive compounds from plants with maximum yield and purity of required phytochemicals. To obtain good extract yield and more charantin content, a combination of solvents and a successful extraction technique is required for efficient extraction of charantin due to its unique structure with a non-polar head and polar tail. In this work, great efforts are done on green approach towards the extraction of natural biomolecule charantin by using the modified SFE technique and its quantification in the extract by HPLC-UV method. Charantin has the potential for the production of novel phytoformulations for the side-effect-free treatment of diabetes and its consequences.

MATERIAL AND METHODS

Collection and Authentication of Plant Material

MC Plants were received from a local region at the flowering stage in the month of September -November from Anewadi village near Satara, Maharashtra, and authenticated by BSI, (Botanical Survey of India) Pune. The unripe fruits of plant were collected dried and coarse powder was prepared using a mechanical grinder and stored for subsequent study. Charantin (purity 99%) standard was purchased from chromaDex, US.

Chemicals and Reagents

Analytical grade chemicals were utilized in research and purchased from Merk, Mumbai. Standard charantin was purchased from ChromaDex US.

Preparation of Herbal Extract

Soxhlet extraction

Coarsely dried powder of fruits of MC (100 gm) was first extracted by using petroleum ether and then exhaustively extracted by using various solvents (400 mL) like ethanol 95%, dichloromethane, chloroform and n-hexane. The extraction was carried out for total 6 hours at temperatures as per the boiling point of solvents (dichloromethane 37°C, 95% ethanol 78°C, and petroleum ether 39.6°C) Both extracts were properly dried by using rota evaporator at 60°C and percentage extract yield was calculated. [28] The ethanol extract gives high yield compared to other solvents and was kept in a dark bottle in the refrigerator for further use.

Supercritical CO_2 extraction

The supercritical carbon dioxide extraction was performed at fixed temperature and pressure of 45°C and 30 MPa, respectively. Dried powder of fruits was placed in a bag and put into an extraction vessel. Pure 99.9% CO_2 gas was liquidized using a chiller bath and driven to an extraction container via a

Table 1: SFE process parameters

Temperature	*45℃*
Pressure	30 Mpa
Flow rate	280 mL/min
Co-solvent/modifier	Ethanol
Separator temperature	40℃

liquid CO_2 pump at a 280 mL/min fixed flow rate. The heater and the chiller were set at 50 and 6°C, respectively. Then, a supercritical pump continually pushed liquid CO_2 from the CO_2 container to the extraction system. After completion of the extraction process, the extract was collected from a separator vessel, sealed and kept in a freezer till further use.[17, 29]

The % extract yield of both the extracts was then calculated as follows,

Extract yield (%) = MC Extract (g)/Initial sample (g) ×100

Physicochemical Evaluation

To assess the efficacy and purity of the medicine, an investigation of the physicochemical constants of bitter gourd fruit powder were conducted. According to WHO recommendations, a number of physicochemical characteristics, including ash value, extractive value, and moisture content, was calculated and data collected from these tests proved helpful for standardization and obtaining quality standards. Table 1 presents the results of the physicochemical evaluation.[30,31]

Preliminary Phytochemical Screening

Phytochemical screening of both the extracts (soxhlet and SC-CO_2) were performed using standard procedures and the results of the same are reported in Table 2.[32,33]

Total Saponin Content (TSC)

The vanillin-sulfuric acid technique was used to estimate TSC of both extracts. It is a frequently used, quick, easy, and affordable approach for identifying and measuring saponins in plant materials. The extract was combined with vanillin (8 w/v%) and sulfuric acid (72 w/v%), and the combination was then heated for 10 minutes at 60°C. Using a spectrophotometer, the reaction mixture exhibits a unique red-purple color at 473 and 560 nm wavelengths. The amount of total saponins was expressed as equivalents of diosgenin. Here diosgenin was used as the reference standard. [34]

Chromatographic Analysis

Thin layer chromatography

Both extracts' TLC was done using silica gel as a stationary phase and compared with standard charantin. Increasing order of polarity of solvents was used as mobile phases for the development of plates on a trial-and-error base. The mobile phase showed maximum separation of phytoconstituents with good resolution (Benzene: methanol-8:2 v/v) was selected for further separation and isolation of charantin.[35,36]

Quantitative analysis by HPLC-UV method

High performance liquid chromatography equipped with ultraviolet-visible detection (Shimadzu Corporation) (HPLC-UV) was utilized to identify and quantify charantin in both extracts. Several mobile phases compositions were investigated during the initial process development to accomplish the successful separation of charantin. The best resolution and sharp peak were obtained using methanol: water (98:2 v/v) at 204 nm. Different concentrations between the 0.1 and 0.5 mg/mL ranges were investigated for the quantitative assessment of charantin content in specific extracts.[37,38]

Table 2: HPLC method parameters

Column	Symmetry C-18
Mobile phase	Methanol: water (98:2 v/v)
Mobile phase flow rate	0.4 mL/min
Wavelength	204 nm
Temperature	25°C
Volume of Injection	5.0 µL

Validation of Developed Method

The method validation was performed in accordance with ICH guidelines for different tests.

System-suitability

To check repeatability, five replicated injections (concentration 0.1 mg/mL) of standard charantin. On the basis of chromatoghram %relative standard deviation (RDS%) between retention time and area under the curve of charantin was calculated by the formula,

RSD = [(SD/mean) × 100] (Should not be more than 2%).

Linearity

Solutions of standard charantin with different concentrations were prepared and a calibration curve was constructed. The linear regression line was applied to ascertain the linearity and concentration of the test samples. Each solution was analyzed in triplicate.

Accuracy

The recovery analyte test studied accuracy at three different concentration levels.[39] A known quantity of sample solution was added at different levels, i.e., 80, 100, and 120%. The suggested technique re-examined the samples and three samples were made for each stage of recovery. Recovery% was used to indicate accuracy.

Precision

By analyzing charantin solution at three distinct concentrations for three different times on similar day, intra-day precision (repeatability) was ascertained. By analysing the same three charantin solutions on three separate days, inter-day precision (intermediate precision) was ascertained.

Determination of Limit of detection and limit of quantification

The lowest quantity of test sample detected in a sample is known as LoD, while the lowest quantity measured in a given sample with accuracy and precision is known as LoQ.

LoD and LoQ of charantin were calculated using calibration standards[40]

LoD = 3.3 × Avg. SD/S

LoQ = 10 × Avg. SD/S
S = Slope
SD = Standard deviation.

Ruggedness

The method ruggedness was determined through analysis of 6 g/mL charantin concentration with the similar instrument by two analysts at same environment and operation settings.

Robustness

Robustness was evaluated by creating minor variations purposefully in the optimized values of the method parameters like wavelength, flow rate, composition of mobile phase etc. and by evaluating their effect on peak area, retention time and % of charantin.

In-vitro Pharmacological Screening

Antioxidant activity by DPPH method

The widely used DPPH method was modified to test the extracts' ability to scavenge free radicals.[41,42] In ethanol, various extracts and ascorbic acid (standard) concentrations were produced (0.2, 0.4, 0.6, 0.8,1,1.2 and 1.6 mg/mL). A 95% ethanol was used for the preparation of DPPH solution. (10 mg in 250 mL ethanol). Three mL DPPH solution were added in 1-mL of each solution of various extract concentrations and incubated at 37°C for about 30 minutes. Then, absorbance was taken by UV-visible spectrophotometer at 517 nm. All samples were assessed in triplicate on each extract. Small value of absorbance showed more free radical scavenging activity. In 95% ethanol and reference standard ascorbic acid, a stable antioxidant were utilized as the blank and positive control, respectively. %inhibition of standard ascorbic acid was also performed using the same procedure, %inhibition was calculated as follows,

$$\% \text{ Radical scavenging activity} = \frac{\text{Absorbance of blank} - \text{Absorbance of sample}}{\text{(\% inhibition) Absorbance of blank}} \times 100$$

The IC_{50} value indicates the antioxidant activity which is the concentration in mL that inhibited 50% production of DPPH free radicals and it was calculated using a graph of %inhibition versus concentration.

Antidiabetic Activity

In-vitro α-amylase inhibition test

Using starch substrate, the DNSA method was used to measure the α -amylase inhibitory activity.[23-43] Various concentrations of extracts and standard acarbose solutions (0.2,0.4, 0.6, 0.8, 1, 1.2 mg/mL) were prepared in DMSO. Reaction mixture containing 1-mL alpha-amylase solution and extracts or standard in various concentration were incubated for 30 minutes at 37°C. Subsequently, 1-mL starch solution in 0.02M phosphate was added to each sample and again incubated for 15 minutes. The reaction was completed by addition of 1-mL of DNSA color substance. The reaction mixtures were incubated for an additional 5 minutes. After 5 minutes, the mixes were cooled at room temperature, and a UV spectrometer was used to measure the absorbance at 540 nm. The formula used to compute the percentage inhibition was,

%Inhibition = [(Ac − As) / Ac] × 100
Ac = Control- Absorbance
As = Sample-Absorbance

%inhibition vs. concentration graph was plotted to calculate the IC_{50} of each extract and standard acarbose.

Alpha-Glucosidase Inhibition Activity

A previously reported method with slight modification was employed to investigate α-glucosidase inhibitory activity of both extracts.[24] In brief, a mixture of 1-mL of each solution of extracts or standard acarbose (0.2, 0.4, 0.6, 0.8, 1, 1.2 mg/mL) prepared in DMSO and 1-mL of α -glucosidase solution (pH 6.8) was incubated for 30 minutes at 37°C. Following the pre-incubation period, 1-mL of p-NPG solution prepared in phosphate buffer of pH 6.8 was mixed with the reaction mixture then again kept incubated for 15 minutes at same temperature. The reaction was completed by the addition of 0.5 molar tris buffer (4 mL) and the absorbance was measured at 405 nm. The % inhibition and value of IC_{50} were computed by a similar method as above.

RESULTS AND DISCUSSION

Extraction of Fruits of MC

The dried powder of MC fruits was extracted by conventional soxhlet extraction and modified SC-CO_2 extraction. The results of extract yield of each extract in percentage are depicted.

Physicochemical Evaluation

Evaluation of physicochemical constants are critical for detecting drug adulteration. Table 3 summarizes the results of several physicochemical properties of the crude drug.

Phytochemical Screening

The results of (Quantitative test) of the MC fruits extracts proved that both extracts contain a variety of bioactive substances with distinct pharmacological effects (Table 4).

Phytochemical Screening (Quantitative test)

Total saponin content

The saponin content measured in terms of total content of saponins in different extracts are summarized in Table 5 in the present work, diosgenin served as standard reference drug and total saponins in both extracts are reported as diosgenin (DA) equivalent (mg DA/g crude extracts) and was estimated by examining the standard curve.

As compare to soxhlet extract, SC-CO_2 extract found more yield of saponin. This is because the supercritical CO_2 extraction technique yields concentrated high-purity extract and hence it is more suitable extraction method to obtain required phytoconstituents with high yield and purity from plant materials. Calibration curve of standard saponin, diosgenin linear equation at $y=0.0557x + 0.3004$, $R^2=0.997$.

Table 3: Physicochemical evaluations of MC fruits

S. No.	*Physicochemical properties*	*MC (Linn.) values in %w/w*
1	Total ash	11.9 ± 1.61
2	Water insoluble ash	3.2 ± 1.46
3	Acid insoluble ash	3.1 ± 1.42
4	Water extractive	2.8 ± 0.84
5	Methanol extractive	8.2 ± 1.23
6	Ethanol extractive	5.2 ± 0.59
7	Dichloromethane extractive	6.7 ± 0.89
8	Loss on drying	9 ± 1.47
9	Foaming index	>100

Table 4: Phytochemical screening extracts of MC fruits by qualitative analysis

S. No	*Phytochemical test*	*Soxhlet extract*	*Supercritical fluid extract*
1	Saponins	+	+
2	Alkaloids	+	+
4	Carbohydrates	+	+
5	Proteins	+	+
6	Tannins	-	-
7	Flavonoids	+	+
8	Terpenoids	+	+
9	Steroids	+	+

(+ indicates presence - indicates absence)

Table 5: Total saponin content in *M. charantia* fruit extract

Extracts by different extraction methods	*Total Saponin content (mg DAE/g)*
Soxhlet extract	98.4
SC-CO_2 extract	136.3

Chromatographic Analysis

Thin layer chromatography

TLC is a most extensively utilized technique for the identification and isolation of compounds. It is simple, cost effective and reliable method. TLC of both the extracts was performed to identify and separate the charantin. Different solvent systems were tried for better resolution between two spots with good Rf value. (Figures 1 and 2, Table 6)

Evaluation of charantin by HPLC

The HPLC-UV method evaluated the amount of steroidal saponins (charantin) in both SC-CO_2 extract and soxhlet extract. It a simple, effective technique for the quantification of phytochemicals in extracts. Results of extractive yield and amount of charantin in both extracts are given in Table 7. HPLC chromatograms are shown in Figures 3 and 4.

Validation of Method

Suitability assessment

To ensure the functionality and repeatability of HPLC technique, system suitability was carried out. Peak area, repeatability, and theoretical plate were calculated and compared. On the basis of results five replicated standard retention time are nearly same, also %relative standard deviation (RSD%) of standard charantin is less than 2%. The reliable results and low RSD% values (Table 8) support the system's suitability for the accurate and repeatable measurement of charantin.

Table 6: Parameters of TLC study

Extract	Soxhlet and SC-CO_2 extract
Stationary Phase	Silica Gel-G
Mobile phase	Benzene: methanol (8:2 v/v)
Visualizing reagent	Anisaldehyde sulphuric acid
Observed Rf value	0.45

Figure 1: TLC of SC-CO_2 extract Rf value of charantin 0.45 (violet color)

Figure 2: TLC of soxhlet extract Rf value of charantin 0.47 (violet color)

Calibration curve and linearity

Various concentration solutions (0.1–0.5 mg/mL) of standard charantin were prepared and calibration curve was constructed. By plotting the calibration curve linear equation of y = 38860x +11997 and correlation coefficient of 0.9996 was obtained. The linear regression data obtained by the calibration curve

Table 7: Extractive yield and amount of charantin in different extracts of MC fruits

Extract	*Consistency*	*Extract Yield (%w/w)*	*Amount of charantin (mg/g)*
Soxhlet extract	Sticky semisolid	41.36	36.24
SC-CO2 extract	Semisolid	18.62	611.52

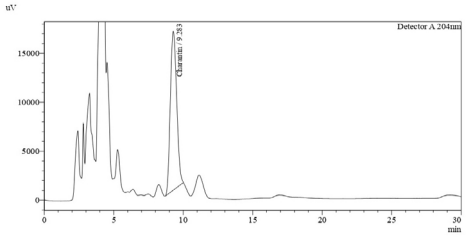

Figure 3: HPLC-Chromatogram of isolated charantin (204 nm)

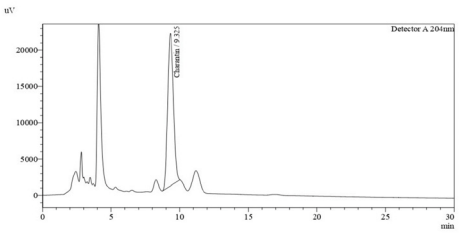

Figure 4: HPLC-Chromatogram of Standard charantin (204 nm)

indicated the good linearity of the method. (Figure 5 and Table 9)

Accuracy

Results of the %recovery for all 3 concentrations ranged from 99.72 to 99.32% with %RSD from 0.23 to 0.41, demonstrating the remarkable accuracy with which even the minutest changes in concentration of drug could be accurately estimated. Hence developed method is accurate, reliable and applicable in routine analysis (Table 10).

Precision

Precision is the amount of variation between measurements made using repeated samples taken from the same homogeneous sample under predefined settings of environments. Tables 11 and 12 indicates the precision results.

Limit of detection (LoD) and limit of quantitation (LoQ)

The LoD and LoQ for charantin were found to be 0.06 and 0.36 µg/mL respectively.

Ruggedness

The concentration of charantin re-covered by two experts using the same method and equipment is shown in Table 13. Based upon it, the percent RSD found to be less than 2% indicates ruggedness of method.

Table 8: Repeatability results

Number of injection	*Retention time of charantin*	*Area of charantin*
1	9.083	621808
2	9.092	618668
3	9.1	616560
4	9.108	611247
5	9.108	618894
Mean	9.0982	617435
STD Dev	0.01077961	3931.573969
%RSD	0.118480693	0.63675861

Table 9: Linearity study results

Concentration (mg/mL)	*Area*				*%RSD*
	1	*2*	*3*	*(Mean ± SD)*	
0.1	48800	48600	48729	48709 ± 0.066	0.834
0.2	74736	74854	74863	74817 ± 0.068	0.686
0.3	146351	146561	146398	146503 ± 0.033	0.932
0.4	218965	218774	218659	218099 ± 0.036	0.853
0.5	364317	364418	364367	364367 ± 0.033	0.538

* Indicates ± SD (n=3)

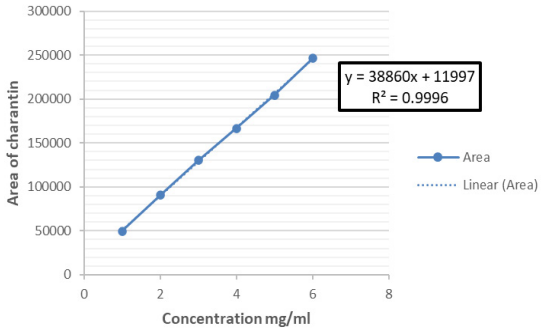

Figure 5: Calibration curve of charantin at different concentrations at 204 nm

Robustness

Robustness of method was evaluated by observing the impact of minor deviations of mobile phase flow rate and wavelength. The results obtained by altering the wavelength and flow rate were matching with those under ideal circumstances. (Table 14). These changes of wavelength and flow rate of mobile phase gives %RSD value less than 2% henceforth the method was verified as robust.

Table 10: Accuracy (recovery) study results

Concentration of Reanalysed Sample (µg/mL)	*Recovery level (%)*	*Quantity of analyte taken (µg/mL)*	*Quantity of analyte Detected (µg/mL)*	*Recovery (%)*	*%RSD*
6 (µg/mL)	80	5	4.86 ± 0.010	98.06	0.41
	100	6	5.97 ± 0.015	99.32	0.38
	120	7	6.76 ± 0.062	97.72	0.23

*Indicates ± SD (n=3)

In-vitro Pharmacological Screening

Antioxidant activity

Hyperglycemia results in oxidative stress, accelerating the development of DM and its complications. Free radical generation rises while antioxidant production falls in diabetes. Therefore, one of the main problems of diabetes is increase in free radical concentration.[44] Antioxidants are therefore always important in the treatment of diabetes.

DPPH free radical scavenging assay

Antioxidants interact with DPPH to change it into 1,1-diphenyl-2-picryl hydrazine, a persistent radical with characteristic absorbance at 517 nm. The reduction in absorption is used to measure the activity of radical scavengers. [45] Table 15 displays the scavenging activity of MC extracts and a comparison to normal ascorbic acid. Figure 6. Depicts the concentration versus scavenging activity graph and estimated IC_{50} value of extracts and ascorbic acid. SC-CO_2 extract (IC_{50} = 25.00 mg mL^{-1}) showed higher activity than Soxhlet extract (IC_{50} = 33.00 mg mL^{-1}).

Table 11: Intra-day precision results

Concentration (µg/mL)	*Area (Mean ± SD)*	*Amt. found (µg/mL)*	*%Amt. found*	*%RSD*
2	74817 ± 0.068	1.93	99.61	0.019
4	146503 ± 0.033	3.76	99.81	0.017
6	218099 ± 0.036	5.92	99.59	0.015

*Indicates ± SD (n=3)

Table 12: Inter-day precision results

Concentration (µg/mL)	*Area (Mean ± SD)*	*Concentration found (µg/mL)*	*%concentration Found*	*%RSD*
2	74917 ± 0.058	1.94	99.82	0.063
4	146703 ± 0.05	3.85	99.61	0.047
6	218399 ± 0.04	5.96	99.89	0.039

*Indicates ± SD (n=3)

Table 13: Ruggedness results

Concentration (µg/mL)	*Analyst*	*Concentration of detected (mean ± SD)*	*Concentration Detected (%)*	*% RSD*
6	Analyst 1	5.964 ± 0.0056	98.862	0.868
	Analyst 2	5.974 ± 0.0058	99.121	0.783

* Indicates ± SD (n=3)

Table 14: Robustness results

Conc. (µg/mL)	Parameters (Change in factors)	Amount detected (mean ± SD)	Amount detected (%)	%RSD
6	Wavelength 278 nm	5.938.1 ± 0.024	99.16	0.720
6	Wavelength 280 nm	5.947.4 ± 0.073	99.36	0.638
6	Flow rate 0.4 mL/min	5.978.5 ± 0.030	99.91	0.648
6	Flow rate 0.6 mL/min	5.987.50 ± 0.019	99.56	0.789

* Indicates ± SD (n=3)

Antidiabetic activity

Modulating glucose absorption is an important therapeutic approach for the management of hyperglycemia. One of the six potential methods for managing diabetes is the regulation of postprandial glucose. Thus, blocking the carbohydrates breakdown enzymes like α -amylase and α -glucosidase in gastrointestinal trac results in the delay in absorption of glucose and reduction in postprandial blood sugar level.[44]

α-Amylase inhibition activity

α-amylase inhibition activity results of MC fruits extract are showed in Table 16. Figure 7 depicts the graphical representation of alpha amylase inhibition activity which helps assess IC50 value of both extracts and standard reference drug. The IC50 value is the amount of extract or reference medication needed to block 50% of the enzyme in a reaction mixture. Both extracts demonstrated inhibitory action that was dosage dependent. Small IC_{50} value relates to larger potency and improved therapeutic effectiveness. SC-CO2 extract exhibited the higher inhibitory activity (IC50 = 0.49 mg mL^{-1}) as compared with soxhlet extract (IC_{50} = 0.62 mg mL^{-1}). Furthermore, the IC_{50} of SC-CO_2 extract is nearly equal to that of standard acarbose, indicates it a good alpha amylase inhibitor. Higher activity of SC-CO_2 extract must be credited for its excellent purity and high concentration of charantin content.

Alpha-glucosidase inhibition activity

Table 17 represents the results of *in-vitro* α- glucosidase inhibitory activity study of MC fruits extract and standard drug acarbos. Figure 8 graphically depicts alpha glucosidase activity and aids in estimating the IC_{50} value of both the extract as well as standard acarbose. SC-CO2 extract displayed maximum α- glucosidase inhibitory activity (IC_{50} = 0.39 mg mL^{-1}) as compared to soxhlet extract. (IC_{50} = 0.52 mg mL^{-1}).

DISCUSSION

Suitable combination of solvents and a successful extraction technique is required for an efficient extraction of charantin to obtain good extract yield and more content of charantin in extract. In the present study extraction of potent antihyperglycemic biomolecule charantin, of *M. charantia* L. was performed using conventional soxhlet extraction and modified supercritical fluid extraction methods and comparison was made in both methods in terms of % extract yield, charantin concentration in both extracts (Soxhlet extract and SC-CO_2 extract) and antidiabetic activity. Both extracts were exposed to detailed phytochemical investigation and results showed both extracts contain saponins, alkaloids, carbohydrates, proteins, flavonoids a major active chemical constituent. The total saponin contents test of both extracts showed, SC-CO_2 extracts contains more saponin (136.3 mg DAE/g) than soxhlet extract (98.4 mg DAE/g).

TLC method confirmed the presence of charantin in given extracts and further, quantitative estimation of charantin in both extracts was done by HPLC technique. According to ICH requirements, the developed HPLC-UV technique

Table 15: Antioxidant activity of soxhlet and SC-CO_2 extracts of MC and ascorbic acid by DPPH method

Concentration of sample	Ascorbic acid	SC-CO_2 extract	Soxhlet extract
0.2	19	14	8
0.4	34	23	14
0.6	57	29	20
0.8	86	42	29
1	90	78	45
1.2	92	86	76
1.4	94	89	80
IC_{50}	0.14	0.25	0.33

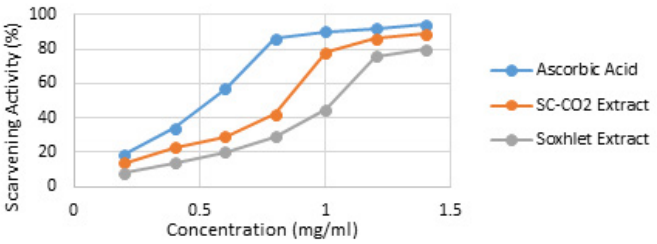

Figure 6: Antioxidant activity and estimation of IC_{50} value of soxhlet and SC-CO_2 extracts of MC and ascorbic acid

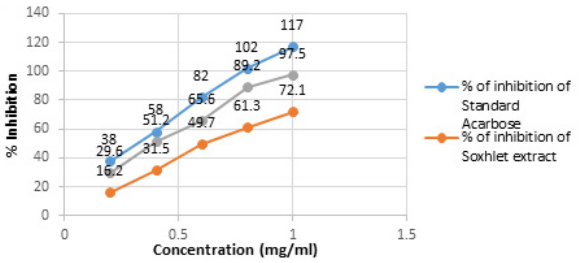

Figure 7: Dose-dependent alpha-amylase inhibitory activity MC fruit extracts and reference standard acarbose

Table 16: In-*vitro* Antidiabetic activity and IC_{50} value of soxhlet and SC-CO_2 extracts of MC by *α*- amylase method

Concentration of sample	*%inhibition of standard acarbose*	*%inhibition of soxhlet extract*	*%inhibition of SC-CO_2 extract*
0.2	38	16.2	29.6
0.4	58	31.5	51.2
0.6	82	49.7	65.6
0.8	102	61.3	89.2
1	117	72.1	97.5
IC_{50}	0.37	0.62	0.49

was verified, and method was proved as correct, specific, linear, and repeatable. As compared with SC-CO_2 extraction (18.62%). Soxhlet extraction gave the highest yield of extract (41.36%). Although soxhlet extraction yielded a more extract yield, charantin content is maximum in SC-CO_2 extract than the soxhlet extract. From the *in-vitro* antidiabetic study it is revealed that both the extracts have antidiabetic activity but when compared to soxhlet extract, the SC-CO_2 extract showed high inhibitory activity on α-amylase and α-glucosidase

Table 17: *In vitro* antidiabetic activity and IC_{50} value of Soxhlet and SC-CO_2 extract of MC by *α*-glucosidase method

Concentration of sample	*%inhibition of standard acarbose*	*%inhibition of soxhlet extract*	*%inhibition of SC-CO_2 extract*
0.2	44.2	24.4	38.6
0.4	65.5	39.5	52.6
0.6	98.3	58.6	78.5
0.8	112.8	68.3	97.3
1	129.3	79.4	105.7
IC_{50}	0.27	0.52	0.39

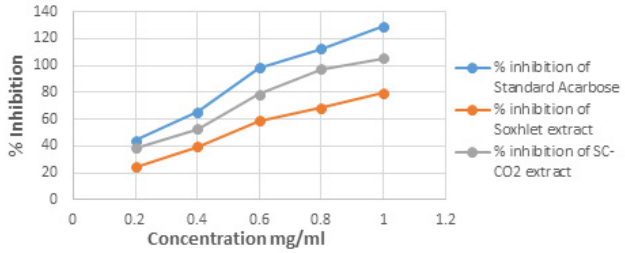

Figure 8: Dose-dependent α-glucosidase inhibitory activity and estimation of IC_{50} of MC extract and acarbose

enzymes indicates more antihyperglycemic activity than soxhlet extract due to high purity extract with more quantity of charantin. Study also indicates that IC_{50} value (0.25 mg/mL) of SC-CO_2 extracts demonstrated more scavenging potential than Soxhlet extract (0.33 mg/mL). All results signified the efficiency of SFE method over conventional soxhlet extraction method. It is most prominent method to extract the bioactive molecules with large quantity and high bioactivity which are beneficial to cure many diseases without any side effects.

CONCLUSION

Here, all revealed data demonstrated the potential of SFE as a method capable of extracting charantin from fruits of *M. charantia* L. with high bioactivity and more quantity. It is the most prominent technology for the extraction of phytochemicals because it has various benefits including short time required for extraction, small quantity of solvent used and high concentration of bioactive compounds. For the quantification of phytoconstituents HPLC-UV technique was established and verified as specified by ICH recommendations and the method was proved as correct, specific, linear, and repeatable. HPLC-UV method is very simple, fast and effective, which can be routinely used to analyze charantin in various vegetable products. *In-vitro* pharmacological screening study revealed that charantin, a steroidal saponin of *M. charantia* possess potent antidiabetic and high antioxidant activities and therefore hold promise for the development of novel herbal formulations to manage diabetes with no any side effects.

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