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SUPERCRITICAL CARBON DIOXIDE EXTRACTION OF MELATONIN FROM *BRASSICA CAMPESTRIS*: *IN VITRO* ANTIOXIDANT, HYPOCHOLESTEROLEMIC AND HYPOGLYCAEMIC ACTIVITIES OF THE EXTRACTS

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Keywords:

Melatonin, Yellow mustard, Supercritical carbon dioxide extraction, Hypocholesterolemic activity, Hypoglycaemic activity

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ABSTRACT: *Brassica campestris* Linn. (yellow mustard), a common Indian condiment, possesses melatonin as an important antioxidant. Optimization of supercritical carbon dioxide extraction (SC-CO_2) process parameters was conducted to obtain an extract having the best combination of yield of melatonin and maximum *in vitro* hypoglycaemic and hypocholesterolemic activities, along with a desirable balance of ω6 to ω3 fatty acids. A full factorial design and response surface methodology were employed for process optimization. Melatonin content of the extracts was obtained by high performance liquid chromatography and by liquid chromatography-mass spectrometry. The optimized extraction conditions were obtained at 300 bar, 50 °C for 120 min. This extract exhibited highest content of melatonin (660.72±41.05 ng/g of dry mustard powder), maximum *in vitro* hypocholesterolemic (60.11±3.89%) and hypoglycaemic (59.70±2.67µg/ml) activities and possessed minimum ω6 to ω3 (1.29) ratio with minimum erucic acid content (2.00±0.20 g/100g of fatty acid). The electron paramagnetic resonance spectroscopy of the extract (0.10±0.001 g/ml) showed 72.10±4.01% scavenging activity of DPPH radicals, establishing its strong antioxidant potency. A new Chrastil equation was developed to compute solubility of melatonin in SC-CO_2 under different extraction conditions. The SC-CO_2 extract of yellow mustard seeds could have promising use as a natural source of melatonin as well as of balanced ω6 to ω3 fatty acids.

INTRODUCTION: *Brassica campestris* Linn., commonly known as field mustard, belongs to the family Brassicaceae. Mustard seeds are principally valued as source of edible oil and also as condiment in culinary; however, the same has been less explored as source of natural antioxidants till date [1]. Ongoing studies in our laboratory have confirmed melatonin as an important antioxidant in yellow mustard (YM) seeds (unpublished data).

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Melatonin (N-acetyl-5-methoxy-tryptamine, an indoleamine), the hormone of darkness, is highly significant as a biopharmaceutical molecule, especially as an antioxidant and also possesses significant hypoglycaemic and hypercholesterolemic properties [2]. Since, melatonin is an easily oxidisable compound [3]; prevention of its oxidative deterioration during extraction from mustard seeds must be averted. Supercritical carbon dioxide (SC-CO_2) extraction technology allows extraction of biomolecules in carbon dioxide and therefore could avert oxidation of melatonin during extraction. Barthet *et al*., in 2002 [4] have employed SC-CO_2 as a non-toxic alternative of harmful organic solvents to extract oil from brown mustard seeds (*Brassica juncea)*.

To the best of our knowledge, extraction of melatonin from yellow mustard seeds by SC-CO_2 has not been reported till date. This high pressure extraction process would most likely rupture the oil glands in mustard seeds and therefore ω6 and ω3 long chain essential fatty acids along with erucic acid (22:1, ω9 fatty acid), are likely to be co-extracted with melatonin.

It is known that the ratio of ω6 to ω3 is an important factor for human health, where an imbalance may lead to adverse health consequences [5]. Simopoulos in 2002 [6], had reported that a higher ratio of ω6 to ω3 (above 20) possibly increased the prevalence of Type II diabetes and cardiovascular disorders in the Indian population, whereas a diet with a lower ratio of the same exerts a suppressive effect on these disorders.

This work therefore aims in obtaining melatonin-rich extracts with low ω6 to ω3 ratio and erucic acid from yellow mustard seeds. The current work focuses on optimization of the SC-CO_2 extraction parameters, such as pressure and temperature using response surface methodology based on the best combination of yield of melatonin and *in vitro* hypoglycaemic and hypocholesterolemic properties of the extracts. It has been assured that the ratio of ω6 to ω3 and erucic acid content are both low in the extracts obtained. This work reports for the first time on the extraction of melatonin as an antioxidant from YM seeds. This work endeavors to obtain a safe 'green' extract of melatonin from YM seeds for favorable end-use of the same as a nutraceutical food supplement.

MATERIAL AND METHODS: B_9 variety of YM seeds (*Brassica campestris*) were provided by Dr. Manas Ghosh (IRDM, Narendrapur, South 24 Parganas, West Bengal, India). The seeds have been cultivated at 22.4386°N, 88.4000°E and at 22 m elevation above mean sea level, under tropical-temperate climate (30-35°C, 75-85% RH) in sandy loamy soil of pH ~ 7-7.5. YM seeds were pulverized using an electric grinder (HL 1618, M/s PHILIPS, India) and the particle diameters (d_p) were determined using the sieve analysis method by screening the seeds through a set of standard sieves in a sieve shaker (AS 200, Retsch, Germany) in accordance with the method reported by Chatterjee and Bhattacharjee, in 2013 [7].

Optimization of SC-CO_2 extraction of melatonin from YM seeds: For SC-CO_2 extraction, a SPE-ED SFE 2 model of M/s Applied Separations (Allentown, USA) was used. The optimization of extraction process parameters (batch size, particle diameter, extraction time and flow rate of gaseous carbon dioxide) was established through several preliminary trials. It was observed that d_p higher than 0.500±0.02 mm decreased the surface to volume ratio of the mustard seed powder and thereby decreased the yield; while a lower particle diameter resulted in tight packing of sample matrix in the extraction vessel restricting free channeling of SC-CO_2 through it and thereby completely impeding recovery of extract.

A batch size greater than 30 g also led to tight packing of the sample matrix and lowered the yield of melatonin in the extract considerably. Therefore batch size of 30 g was maintained in all extractions. Preliminary trials conducted at three different extraction time (90, 120 and 150 min), revealed that yield of melatonin was maximum at 120 min. Therefore the extraction time was fixed at 120 min (static time: 60 min, dynamic time: 60 min).

In the present study, SC-CO_2 extraction parameters were optimized using a 3^2 full-factorial design. Extraction pressures of 200, 300 and 400 bar and temperatures of 40, 50 and 60 °C were employed. The extracts were collected in 25 mL glass vials placed in an ice bath in the dark. They were weighed and filtered through a syringe filter (0.22 µm) and stored in amber colored screw capped glass vials in an inert atmosphere of nitrogen at -20°C in the dark until further analyses.

Quantification of melatonin in mustard seed extracts: The filtrates of YM seed extracts were subjected to quantitative HPLC (High Performance Liquid Chromatography) analysis [8]. The HPLC system (M/s JASCO, Easton, US) consisted of a reversed-phase C_{18} column (L×I.D. 25 cm×0.46 cm) adjusted with a HPLC pump (M/s JASCO PU 2080 plus, Easton, US) and an injector with a 20 µL sample loop. Retention time (RT) of melatonin was determined using HPLC-PDA detector (M/s JASCO MD 2015 plus, Easton, US) at 280 nm. The mobile phase comprising of water with 20% acetonitrile, was purged at a constant flow rate of 1 mL/min in isocratic mode.

A standard curve of melatonin was prepared by dissolving a pure standard of melatonin sample in water-methanol (1:1 v/v). Dilutions were made immediately before injection into HPLC. Each solution was injected three times and the mean peak areas were considered for the preparation of standard curve. Amount of melatonin present in the extracts was quantified from the standard curve.

To confirm whether the peak with retention time 10.50 - 11.50 min obtained in HPLC-PDA, was due to melatonin, further validation studies were conducted using liquid chromatography-mass spectrometry (LC-MS/MS) (M/s Applied Biosystem and MDX SCIEX, API 2000, Canada, USA) in a C_{18} column (L×I.D. 5 cm×5 µm), according to the method reported by Kocadağlı *et al*., in 2014 [9]. MS data were acquired in the positive mode and melatonin was identified in multiple reaction monitoring (MRM).

Evaluation of *in vitro* hypoglycaemic activity of YM seed extracts: SFE extracts of YM seeds were subjected to assay of *in vitro* α-amylase activities[10]. The α-amylase inhibition was expressed as IC_{50} value and calculated by the following equation (1):

$$\% \text{ inhibition} = 100 - \{[(Abs)_{test} / (Abs)_{control}] \times 100\} \quad \text{..... (1)}$$

where, $(Abs)_{control}$ is the absorbance of the control sample (containing all reagents except the test sample) and $(Abs)_{test}$ is the absorbance of the test sample.

Evaluation of *in vitro* hypocholesterolemic activity of YM seed extracts: The inhibition of cholesterol in micellar solution is used as an *in vitro* test of hypocholesterolemia [11]. An experimental control was prepared without extract. The percentage micellar solubility inhibition was calculated using the following equation (2):

$$\% \text{ micellar solubility inhibition} = \{1-(cholesterol_{test}/cholesterol_{control})\}\times 100 \quad \text{... (2)}$$

The supernatants of micellar solutions were subjected to HPLC-PDA analysis at 200 nm for determination of their cholesterol contents, adopting the method of Oh *et al*., in 2001[12].

Analysis of morphology of YM seed matrix by scanning electron microscope (SEM): SEM analyses were conducted for ground and sieved YM seed matrices before and after SC-CO_2 extraction. The samples were placed on a clear glass, then vacuum-dried by vacuum evaporator and coated with platinum using a Autofine Coater (JFC-1600, JEOL Company Ltd., Japan) and analyzed using field emission scanning electron microscope (FESEM) (JSM-6700F, JEOL Company Ltd., Japan) operated at 5 kV, at a working distance of 8 mm.

Evaluation of phytochemical property of YM seed extracts: Extracts obtained from different conditions of SC-CO_2 extraction were subjected to phytochemical characterization, such as for evaluation of antioxidant activity (by DPPH radical scavenging activity and expressed as IC_{50} value), total phenolic content (expressed as µg gallic acid equivalent/g of dry mustard powder) and reducing power (µg of BHT/g of dry mustard powder) [13].

Electron paramagnetic resonance (EPR) spectroscopy: The antioxidant activity of the YM seed extract obtained at the optimized condition of SC-CO_2 extraction was evaluated by EPR spectroscopy. 200 µl test sample was added to 200 µL of DPPH (2 mM) solution. After shaking vigorously for 10 s, the solution was transferred into the EPR quartz tube (I.D. 4.0 mm) and inserted into the microwave cavity (JEOL X-BAND microwave unit, JEOL Ltd., Japan) of the EPR spectrometer (JES-FA 200 ESR Spectrometer, JEOL, Japan). The EPR spectrum was recorded after 30 s at room temperature (25±1 °C) using X-band frequency of 9.65 GHz [14]. The radical scavenging activity of the extract was calculated by the following equation (3):

$$\% \text{ DPPH scavenging} = [(I_0 - I) / I_0] \times 100 \quad \text{..... (3)}$$

where: I_0 = intensity of DPPH signal. I = integral intensity of the DPPH signal after addition of YM extract.

Analysis of fatty acid composition of YM seed extracts by gas chromatography-flame ionization detector (GC-FID): The fatty acid methyl esters (FAME) of fatty acids in SC-CO_2 extracts of YM seeds were prepared in accordance with the method reported by Ghosh *et al.*, in 2014 [15]. Analysis of the FAME samples was conducted

by GC in Trace GC 700 system (Thermo Scientific, USA) equipped with a TR-1 capillary column (L×I.D. 30 m × 0.32 mm, 0.25 μm film thickness) and FID. The injector and detector temperatures were 250 ºC and 260 ºC, respectively. The oven temperature was programmed as follows: 60 ºC (2 min hold), 60 ºC to 200 ºC at 10ºC/min and final hold at 260 ºC (8 min). 1 μL of FAME sample (dissolved in *n*-hexane) was injected into GC in splitless mode for analyses. 37-component FAME mix [butyric acid methyl ester (C4:0) − nervonic acid methyl ester (C24:1, ω9)] of Supelco Analytical, MO, USA was used as the standard.

The fatty acid composition of the mustard seed extract was determined by performing FAME-GC-FID of the oil extracted from the seeds by Soxhlet extraction.

Statistical analyses: All experiments were conducted in triplicate and the data were expressed as means ± SD of three independent experimental runs. Statistical analysis of the data was conducted by one-way analysis of variance (ANOVA), response surface analysis (RSM) and regression modeling. Significant differences between means were determined by Duncan's multiple-range test. A p value of ≤0.05 was used to verify the significance of the tests. In the present study, STATISTICA 8.0 software (Statsoft, Oklahoma, USA) was used to test the experimental results.

Determination of solubility of melatonin in SC-CO_2: The solubility of melatonin in different SC-CO_2 extraction conditions was calculated [16] by the ratio of the total mass of extracted melatonin (g) to the mass of CO_2 consumed in the extraction process as shown below:

y= mass of melatonin/ mass of CO_2 = Mo/ρV ... (3)

where, Y is the solubility (mass fraction) of melatonin in SC-CO_2 extracts, Mo is the total mass of melatonin extracted (g), V is the volume of CO_2 (mL) used for extraction and ρ is the density of SC-CO_2 (kg/m^3) under different extraction conditions. The densities of SC-CO_2 under different extraction conditions (temperature, pressure) were calculated using empirical Peng-Robinson cubic equation of state as has been reported by Ghosh *et al*., in 2016 [17].

Evaluation of solubility of melatonin in SC-CO_2 using Chrastil equation: Chrastil equation gives a linear relationship between the logarithm of solubility of a solute and the logarithm of SC-CO_2 density and is represented as[17].

$$\ln S = k \ln \rho + F + G/T \text{…….(4)}$$

where, 'S' is the solubility of melatonin in the gas phase (g/kg), k is the association constant related to the total number of molecules in the complex[17], ρ is the density of CO_2 (kg/m^3), F and G are empirical constants in density correlation and T is the temperature (K).

The values of k, F and G of Eq.4 [17] were determined and a linear equation was developed which allows the prediction of solubility of melatonin in SC-CO_2 under varying extraction conditions.

RESULTS AND DISCUSSION:

Total yields of extracts: The yields of the extracts (mg/g of dry YM powder) obtained from YM seed powder by SC-CO_2 under different extraction conditions have been tabulated in **Table 1**. It was observed that maximum yield of extract from YM seeds having detectable amount of melatonin (103.09±6.61 mg/g of dry mustard powder).was obtained at 50 °C, 300 bar, 120 min.

Optimization of SC-CO_2 extraction parameters using response surfaces: Table 1 represents the melatonin contents of SFE extracts of YM under varying extraction conditions. The maximum yield of melatonin (660.72±41.05 ng/g of dry mustard powder) was obtained at 50 °C, 300 bar, 120 min (extract coded as YM best). This was owing to the fact that solubility of melatonin was maximum at these extraction conditions (discussed later). The above extract also showed maximum α-amylase inhibition activity along with maximum inhibition of micellar solubility of cholesterol (**Table 1**).

From the ANOVA of regression model, it was established that yield of melatonin increased significantly (p=0.00) with increasing extraction temperature from 40 °C to 50 °C, at constant extraction pressure.

TABLE 1: EXPERIMENTAL YIELDS OF MELATONIN FROM YELLOW MUSTARD SEEDS BY SC-CO_2 EXTRACTION

Total no of runs	Extraction pressure (Bar)	Extraction temperature (°C)	Total extraction time (min)	Total yield of extract (mg/g of dry mustard powder)[a]	Melatonin content (ng/g of dry mustard powder)[a]	50 % α-amylase inhibitory concentration (µg/ml)[a]	Cholesterol miceller solubility inhibition (%)[a]
1	200	40	120	15.35±1.05	56.44±4.50	72.33±3.50	45.23±3.45
2	200	50	120	53.59±3.57	197.03±11.55	91.00±2.22	56.40±2.56
3	200	60	120	19.11±1.13	70.26±4.50	111.67±4.50	39.38±1.67
4	300	40	120	62.07±3.66	400.48±24.36	62.00±2.55	43.78±2.24
5	300	50	120	103.09±6.61	660.72±41.05	59.70±2.67	60.11±3.89
6	300	60	120	126.64±1.90	251.34±12.90	78.00±4.29	49.55±3.49
7	400	40	120	29.89±1.25	251.26±10.50	71.00±3.22	45.59±2.24
8	400	50	120	69.95±4.70	592.45±34.50	62.23±2.87	59.52±1.67
9	400	60	120	165.38±3.50	330.23±15.50	71.37±5.43	47.37±2.12

[a]Total yield of extract, melatonin content, 50 % α-amylase inhibitory concentration, Cholesterol miceller solubility inhibition of dry powder of mustard seed are mean ±SD of three independent extraction of three batches of yellow mustard seeds

However, the yield of melatonin did not change significantly (p= 0.53) with increasing extraction pressure from 200 to 400 bar. From the ANOVA study, it can be concluded that two-level interaction factor (temperature-pressure), did not have significant effect (p=0.38) on the yield of melatonin.

Extraction of melatonin mandatorily requires few steps of purification of the extract [2]. Purification is known to be responsible for production of high level of oxidants, such as H_2O_2 and other free radicals which may lead to destruction of melatonin. In our study, the SC-CO_2 extraction parameters were tuned to achieve selective extraction of melatonin, eliminating the need for cumbersome downstream purification of the extracts. This justifies obtaining maximum yield of melatonin by SC-CO_2 extraction technology.

The YM best extract also exhibited minimum IC_{50} value (59.70±2.67 µg/ml) for α-amylase inhibition and maximum inhibition for micellar solubility of cholesterol (60.11±3.89%), indicating maximum hypoglycaemic and hypocholesterolemic activity respectively.

Generation of response curves: The effects of extraction pressure and temperature on the yields of melatonin, IC_{50} values of α-amylase inhibition of the extracts and % inhibition of micellar cholesterol solubility of the extracts, are shown in **Fig. 1A, 1B** and **1C**, respectively. Regression modeling was used for characterization of the response surfaces.

Regression Modeling: Regression modeling was conducted by generating second order polynomial equations for response as a function of extraction temperature and pressure. The second order polynomial equation that fitted our experimental variables is stated below.

$$Y = B_0 + \Sigma B_i X_i + \Sigma B_{ii} X_i^2 + \Sigma B_{ij} X_i X_j \quad \text{..... (5)}$$

where, Y represents the experimental response [yield of melatonin (Eqn. 6), inhibition of IC_{50} value of α-amylase inhibition (Eqn. 7) and micellar solubility of cholesterol (Eqn. 8)] B_0, B_i, B_{ii}, and B_{ij} are constants and regression coefficients of the model; X_i and X_j are two independent variables in coded forms. The expanded model includes linear, quadratic and cross-product terms as shown below (with intercept):

$$Y = -7634.55 + 11.88X_1 - 0.02X_1^2 + 250.90X_2 - 2.57X_2^2 - 0.02X_1X_2 \quad \text{..... (6)}$$

$$Y = 136.652 - 0.326X_1 + 0.001X_1^2 - 1.132X_2 + 0.049X_2^2 - 0.009X_1X_2 \quad \text{.....(7)}$$

$$Y = -200.012 + 0.341X_1 - 0.001X_1^2 + 8.151X_2 - 0.085X_2^2 - 0.002X_1X_2 \quad \text{.....(8)}$$

where, X_1 and X_2 are extraction pressure and temperature, respectively. The above equations (5, 6, and 7) explain the effects of X_1 and X_2 on the response Y.

The effects of the above parameters and their interactions were evaluated. It was observed that

extraction temperature both in linear and quadratic forms (p=0.00) showed significant effects on the yields of melatonin. It was found that pressure (p=0.00), temperature (p=0.00) and the two-level interaction factor (p=0.00) had significant effects on the α-amylase inhibitory activities of YM seed extracts. Extraction pressure (p=0.08) and temperature (p=0.82) in linear forms had no effect on inhibition of micellar solubility of cholesterol but their second-order terms (X_1^2, X_2^2) had significant effects (p=0.00 for both) on the same. These findings suggest that the extract has the potential to be utilized as a hypoglycaemic and hypocholesterolemic agent. The plots of observed with the predicted values showed a close fit. Thus, a statistically significant multiple regression relationship [r = 0.95 for yield of melatonin, r =0.98 for IC_{50} value of α-amylase inhibition and r = 0.81 for inhibition of micellar solubility of cholesterol] among the independent variables and the responding variables could be established. The complete quadratic model showed a very good fit.

Analysis of response surfaces: Form the test statistics for the regression models discussed above, it was observed that extraction temperatures had significant effects on yields of melatonin and on the α-amylase inhibition activities of SC-CO_2 extracts of YM seeds.

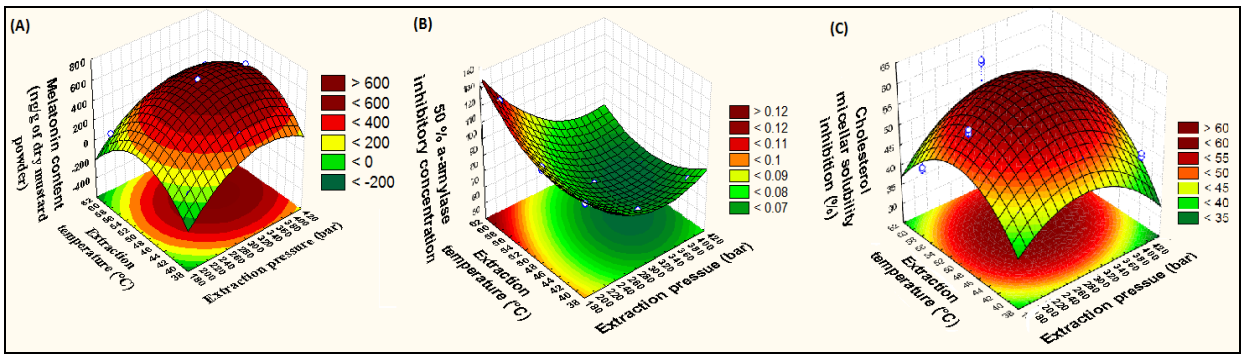

FIG. 1: RESPONSE SURFACES INDICATING (A) YIELD OF MELATONIN (B) 50 % α -AMYLASE INHIBITORY CONCENTRATION (C) CHOLESTEROL MICELLAR SOLUBILITY INHIBITION (%); AS A FUNCTION OF EXTRACTION TEMPERATURE (40, 50, 60 °C) AND PRESSURE (200, 300, 400 bar) AT 120 min (STATIC+ DYNAMIC) WITH FLOW RATE OF 2 l/min FOR 30 g BATCH SIZE

Optimal processing condition: To determine the optimal processing conditions of extraction of melatonin from YM seeds, the first partial derivatives of the regression equation was conducted with respect to X_1 and X_2 and set to zero. This was done by putting the second order regression equation in matrix form [17]. The point thus obtained is known as the stationary point, X_S (X_{1S}=337.67 bar, X_{2S}=49.93 °C). The melatonin content of the extract, IC_{50} value of α-amylase inhibition and inhibition of micellar solubility of cholesterol predicted at this stationary point were 635.39 ng/g of dry mustard powder, 60.42 µg/ml and 60.28 % respectively; whereas, the actual experimental values obtained were 660.72±41.05 ng/g of dry mustard powder, 59.70±2.67 µg/ml and 60.11±3.89 % respectively; suggesting a close fit of the model.

Characterization of response surfaces: The response curve is characterized by determining whether the stationary point obtained above is a point of maximum response, minimum response or a saddle point. For this purpose, the regression equation was transformed to the canonical form and the eigen values were determined in accordance to the method described by Chatterjee and Bhattacharjee, in 2013 [16]. Since the eigen values obtained in case of yield of melatonin (-0.018790 and -2.56730) and inhibition of micellar solubility of cholesterol (-0.000713 and -0.085293) were negative, X_S was a point of maxima. The eigen values obtained in case of IC_{50} value of α-amylase inhibition were 0.050316 and 0.000677. Since the eigen values obtained were positive, X_S was a point of minima. This extraction condition was considered as the optimized condition since at this condition, the extract exhibited the best combination of highest melatonin yield, maximum hypocholesterolemic activity and maximum α-amylase inhibition activity.

Solubility of melatonin using Chrastil equation: The experimental solubility of melatonin in SC-CO_2 was found (**Table 2)** to increase significantly with increase of temperature from 40 ºC to 50 ºC in the high pressure regime (200-400 bar). From the values of the solubility of melatonin, a linear regression equation was developed according to the Chrastil equation, as shown below.

$$\ln S = 6.31 \ln \rho - 4360.32 /T - 43.09 \text{.....(9)}$$

TABLE 2: DENSITY OF SC-CO_2 AND EXPERIMENTAL SOLUBILITY OF MELATONIN DETERMINED UNDER DIFFERENT EXTRACTION CONDITIONS

Pressure (bar)	Temperature (°C)	Density of CO_2 (kg/m^3)[a]	Solubility of melatonin [g /(kg CO_2)]
200	40	829.00	0.2×10^{-6}
200	50	762.00	0.7×10^{-6}
200	60	692.00	0.2×10^{-6}
300	40	928.00	0.1×10^{-5}
300	50	879.00	0.2×10^{-5}
300	60	830.00	0.9×10^{-6}
400	40	992.00	0.8×10^{-6}
400	50	952.00	0.2×10^{-5}
400	60	912.00	0.1×10^{-5}

[a]Density of CO_2 is calculated from Peng Robinson Cubic Equation of state

The regression coefficient *r* obtained using this equation was 0.75, and the standard error of the equation was 0.59. From this Chrastil equation, logarithm of calculated solubility was plotted against logarithm of SC-CO_2 density [16]. The plots were linear and the isotherms at 40 ºC, 50 ºC and 60 ºC were parallel to each other which affirmed the suitability of Chrastil equation for computing solubility of melatonin in SC-CO_2. The solubility values of melatonin calculated at 40 ºC and 50 ºC were in fair agreement with the solubility values predicted by Chrastil equation. In addition, the solubility of melatonin predicted at 60 ºC by Chrastil equation was found to be higher than the calculated value. It may be owing to the fact that temperature higher than 50 ºC led to co-extraction of some waxy material, which have inhibitory effect on the extraction of melatonin and impeded accurate quantification of the same in the extract. Therefore, Eq. 9 can certainly be used to predict the solubility of melatonin in SC-CO_2 condition up to 50 °C.

Analysis of morphology of YM seed matrix by SEM: In our study, the oil-bearing glands of the seed matrix of YM seeds swelled and ruptured under SC-CO_2 temperature-pressure regimes resulting in release of melatonin from the damaged cells along with the oil therein. This was confirmed from the SEM images of pre-extracted and post-extracted YM seed matrices (**Fig. 2**). The oil diffuses out on the surface of the mustard seed particles and forms a film which eventually solubilizes into the bulk phase of SC-CO_2. Therefore, all the melatonin-rich extracts were therefore oily in nature. This phenomenon has also been reported for SC-CO_2 extraction of methyl eugenol from tuberose flowers[17].

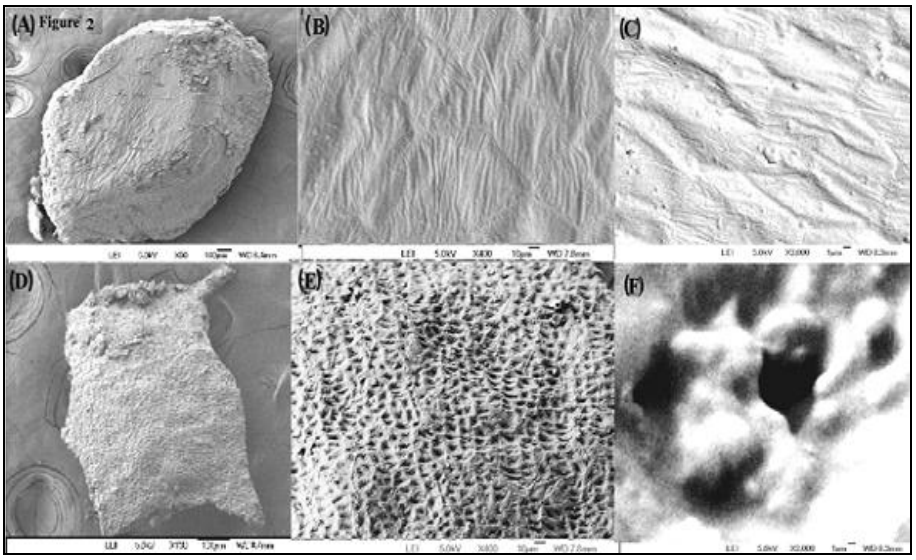

FIG. 2: SCANNING ELECTRON MICROSCOPE IMAGES OF THE SURFACE OF A YM SEED PARTICLE; PRE-EXTRACTION MATRIX (A) 100X MAGNIFICATION (B) 400X MAGNIFICATION (C) 3000X MAGNIFICATION; POST EXTRACTION MATRIX (D) 100 X MAGNIFICATION (E) 400X MAGNIFICATION (F) 3000X MAGNIFICATION

Phytochemical activities of mustard seed extracts: Analyses of phytochemical properties of the extracts are presented in **Table 3**. YM_{best} showed maximum reducing power (365.03±21.2 µg BHT/g of dry mustard powder) along with maximum antioxidant activity (IC_{50} value of DPPH radical scavenging activity was 2.86±1.29 mg/mL. Although, this extract possessed very low amount of phenolic compound (10.33±0.50 µg of GAE/g of dry mustard powder), it exhibited maximum antioxidant potency. This finding possibly established melatonin to be the predominant antioxidant in the YM_{best} extract. Therefore this extract could be certainly considered as a 'melatonin-rich extract'.

TABLE 3: PHYTOCHEMICAL PROPERTIES OF SC-CO_2 EXTRACT OF YELLOW MUSTARD SEEDS

Pressure (bar)	Temp. (°C)	Time (min)	(IC_{50} value of DPPH radical scavenging activity was mg/ mL)[a]	Total phenolic content (µg of GAE/g of dry mustard powder)[a]	Reducing power (µg of BHT/g of dry mustard powder)[a]
200	40	120	16.26±1.30[a]	60.89±2.20[a]	80.50±0.04[a]
200	50	120	14.26±1.30[a]	11.55±0.50[b]	161.25±0.50[b]
200	60	120	16.26±1.30[a]	20.88±1.50[c]	156.25±0.50[c]
300	40	120	9.30±0.30[b]	39.47±2.25[d]	102.38±5.20[d]
300	50	120	2.86±1.29[c]	10.33±0.50[e]	365.03±21.2[e]
300	60	120	5.29±0.20[d]	20.88±1.50[c]	231.03±21.2[f]
400	40	120	8.30±0.30[b]	21.47±0.25[f]	221.03±21.2[g]
400	50	120	7.86±1.29[c]	10.65±0.50[e]	255.03±21.2[h]
400	60	120	6.58±1.15[d]	12.65±0.40[g]	259.03±11.4[h]

[a]IC_{50} of DPPH, total phenolic content, reducing power of dry powder of yellow mustard seed are mean ±SD of three independent extraction of three batches of yellow mustard.
[a, b, c, d, e, f, g, h,] Different letters in a column indicate significant differences at $p < 0.05$ level.

EPR analysis of YM seed extract: The antioxidant activity (by DPPH) of YM_{best} was further validated by EPR spectroscopy. The reduction in the intensity of the DPPH (blank) signal was found to be due to the free radical scavenging activity of the extract (**Fig. 3**). For SC-CO_2 extract (0.10±0.001 g/ml), the DPPH radical scavenging activity was found to be 72.10±4.01%. These findings indicate that this extract could be used as a natural source of melatonin and therefore as an antioxidant.

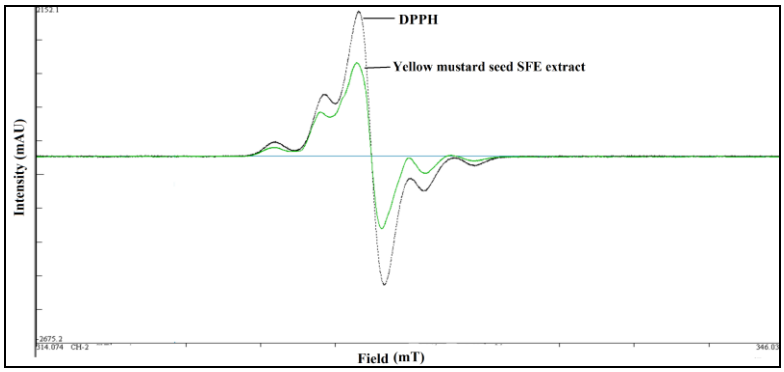

FIG. 3: EPR SPECTRUMS OF DPPH AND DPPH WITH SC-CO_2 EXTRACT OF YM SEEDS

Fatty acid analyses of SFE and Soxhlet extract of YM seed: It was found that the ratio of ω6 to ω3 was 0.89 for the Soxhlet extract and 1.29 for the SC-CO_2 extract (**Table 4**). The ratio of ω6 to ω3 in mustard oil should be in the range of 1 to 2 for Indian population[6]. Therefore we conclude that the melatonin-rich SC-CO_2 extract of YM seeds would not adversely affect human health due to proper balance of ω6 and ω3 fatty acids in the same. This study showed that the erucic acid content (2.00±0.23 g/100g of fatty acid) in the SC-CO_2 extract was lower, vis-à-vis that (2.79±0.11 g/100g of fatty acid) in the Soxhlet extract. This further attested the benefit of selective extraction of target biomolecules (with lower co-extractants such as erucic acid in this case) achieved by SC-CO_2 extraction.

TABLE 4: FATTY ACID PROFILE OF SOXHLET AND SFE EXTRACTS

Fatty acid carbon no	Name of fatty acids	Soxhlet extract (g/100g of fatty acid)[a]	SFE extract (g/100g of fatty acid) [a]
C6:0	Caproic acid	0.61±0.01	6.75±0.21
C8:0	Caprylic acid	3.78±0.02	5.88±0.44
C10:0	Capric acid	2.70±0.11	-
C11:0	Undecanoic acid	-	1.69±0.01
C12:0	Lauric acid	4.98±0.12	4.20±0.25
C13:0	Tridecanoic acid	20.00±1.01	1.42±0.06
C14:1	Myristoleic Acid	4.20±0.22	2.39±0.07
C15:1	Cis-10-Pentadecenoic acid	4.56±0.32	2.27±0.09
C16:0	Palmitic acid	3.71±0.21	-
C16:1	Palmitoleic acid	5.42±0.42	5.66±0.45
C17:0	Heptadecanoic acid	-	1.37±0.08
C17:1	Cis-10-Heptadecenoic acid	2.69±0.11	4.59±0.67
C18:1	Oleic acid	10.14±0.91	4.24±0.23
C18:2	Linoleic acid	4.06±0.12	2.74±0.12
C18:3	Linolenic acid	4.55±0.16	3.54±0.15
C20:0	Arachidic acid	2.04±0.11	0.81±0.01
C20:1	Cis-11-Eicosenoic acid	3.26±0.09	2.59±0.11
C20:2	Cis-11, 14-Eicosadienoic acid	9.97±0.72	10.30±0.52
C20:3	Cis-8,11,14-Eicosatrienoic acid	-	6.42±0.29
C22:1	Erucic acid	2.79±0.11	2.00±0.02
C22:2	Cis-13, 16-Docosadienoic acid	10.56±0.51	5.15±0.03
C22:6	Docosahexaenoic acid	-	3.68±0.04
Others fatty acids		-	22.28±0.19
SFA		37.82±2.23	24.23±0.18
MUFA		33.04±2.44	47.22±0.23
PUFA		29.13±1.12	28.54±0.11
ω6/ω3 ratio		0.89±0.01	1.29±0.02

[a]Fatty acid profile of SFE and soxhlet extracts of yellow mustard seed are mean ±SD of three independent extraction of three batches of yellow mustard.

CONCLUSIONS: This study has established yellow mustard seeds to be a prominent source of the sleep hormone, melatonin. This is the first study on SC-CO_2 extraction of this significant biomolecule from Brassica family. A Chrastil equation has been developed to compute solubility of melatonin in SC- CO_2 under different extraction conditions, which would aid in scale up of the extraction process. The melatonin-rich extract obtained has potential hypocholesterolemic and hypoglycaemic activities. Furthermore, the extract has a well balanced ratio of ω6 to ω3 fatty acids and low erucic acid content (2.00±0.02%). This SC-CO_2 extract of YM seeds could have promising use as a natural source of melatonin and therefore *in vivo* studies on the efficacy of the extract on type II diabetes and hypercholesterolemia are underway.

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