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SFE as a Superior Technique for Extraction of Eugenol-Rich Fraction from *Cinnamomum tamala* Nees (Bay Leaf) - Process Analysis and Phytochemical Characterization

Sudip Ghosh, Dipanwita Roy, Dipan Chatterjee, Paramita Bhattacharjee, Satadal Das

***Abstract*—**Highest yield of eugenol-rich fractions from *Cinnamomum tamala* (bay leaf) leaves were obtained by supercritical carbon dioxide (SC-CO_2), compared to hydro-distillation, organic solvents, liquid CO_2 and subcritical CO_2 extractions. Optimization of SC-CO_2 extraction parameters was carried out to obtain an extract with maximum eugenol content. This was achieved using a sample size of 10g at 55°C, 512 bar after 60min at a flow rate of 25.0 cm^3/sof gaseous CO_2. This extract has the best combination of phytochemical properties such as phenolic content (1.77mg gallic acid/g dry bay leaf), reducing power (0.80mg BHT/g dry bay leaf), antioxidant activity (IC_{50} of 0.20mg/ml) and anti-inflammatory potency (IC_{50} of 1.89mg/ml). Identification of compounds in this extract was performed by GC-MS analysis and its antimicrobial potency was also evaluated. The MIC values against *E. coli*, *P. aeruginosa* and *S. aureus* were 0.5, 0.25 and 0.5mg/ml, respectively.

***Keywords*—**Antimicrobial potency, *Cinnamomum tamala*, eugenol, supercritical carbon dioxide extraction.

I. Introduction

CINNAMOMUM tamala Nees commonly known as bay leaf, belongs to the family *Lauraceae* and is native to South-east Asia, Pacific Islands and Australia, growing mainly in the tropical rain forests at varying altitudes. It has considerable nutraceutical properties, namely antibacterial [1], anti-inflammatory [2] and antioxidative [3], attributed to the presence of compounds chiefly, eugenol, methyl eugenol, β-caryophyllene, α-pinene, β-sitosterol, caryophyllene oxide and cinnamyl acetate [4]-[6].

Plant extracts are commonly obtained by steam distillation and solvent extraction which pose problems of thermal degradation, hydrolysis and water solubilization of desirable constituents, to state a few [7]. Also, the presence of residual solvents in the extracts are causes of environmental and health concerns. The green technology of supercritical carbon dioxide extraction (SC-CO_2) offers a more preferred alternative extraction technique over these conventional extraction procedures. SC-CO_2 extraction technique has been employed by several researchers for extraction of biological important compounds from *Laurusnobilis* leaves [8]-[13]. Most investigations were performed to study the effect of SC-CO_2 extraction parameters - temperature, pressure and extracting time, on the yield of extract from the leaves. To the best of our knowledge, we could not find any report on SC-CO_2 extraction of *Cinnamonum tamala.*

This paper reports for the first time on SC-CO_2 extraction of eugenol-rich fraction from dried bay leaves (*Cinnamonum tamala*) of West Bengal origin (East India). Eugenol has been chosen as the target compound since it is known to be one of the major components of *Cinnamonum tamala* leaves having numerous therapeutically active properties [6], [14]. Although fractional separation model has been advocated for extraction of essential oil from herbaceous matters [15]-[17], eugenol along with several nutraceutical compounds (such as β-sitosterol) is also present in the oleoresin fraction of bay leaves [14]. Therefore we envisage that a eugenol-rich fraction of bay leaves will be a promising therapeutically active extract.

In our investigation, eugenol-rich extracts of Indian variety of bay leaves were obtained by SC-CO_2 extraction, *vis-à-vis* other extraction processes such as liquid CO_2, subcritical CO_2, hydro-distillation and solvent extractions. A central composite rotatable design was employed to design the extraction process of SC-CO_2 to obtain eugenol-rich extracts. All the extracts were subjected to assays of their phytochemical (therapeutic) properties, chiefly for their phenolic content, antioxidant potency, anti-inflammatory activity and reducing power. The extract containing the best combination of eugenol content and phytochemical properties was characterized for its chemical constituents by GC/MS and for its antimicrobial properties, against selected strains of microorganisms.

II. Materials and Methods

A. Materials

Bay leaves (*Cinnamomum tamala* Nees) were purchased from a local market of Jadavpur area in Kolkata, West Bengal, India. The sample was authenticated from the Department of Botany, Ballygunge Science College, Calcutta University, Kolkata, India. Speciality chemicals such as eugenol (99% pure), 1,1-diphenly-2-picrylhydrazyl (DPPH), sodium nitroprusside, Griess reagent and gallic acid were procured

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from M/s Sigma, India. Folin-Ciocalteu's phenol reagent (FCR), methanol, sodium carbonate were procured from M/s E-Merck (India). All chemicals, solvents and buffers used in this work were of AR grade.

B. Preparation of Bay Leaf Powder Samples

The bay leaves were ground to powder in a mixer grinder (Philips Mixer Grinder, Model No- HL 1618, Philips India Limited, Chennai, India). The mean particle diameter of the bay leaf powder was determined to be 0.059cm, using a sieve analysis methods by screening the powdered samples on a sieve shaker through a set of standard sieves (5, 10, 14, 20, 24 and 44 Tyler meshes) [18]. The moisture content of the powder was estimated to be 10% on a dry weight basis, by AOAC method 930.15 [19].

C. Carbon Dioxide Extraction of Bay Leaf Powder

Eugenol-rich compounds from bay leaf powder were extracted by liquid CO_2 in accordance to the method described by McKenzie et al. [20], with modifications. 10g of ground bay leaf powder was subjected to extraction using polypropylene centrifuge tubes provided with plug seal caps.

For subcritical and SC-CO_2 extractions, a SPE-ED SFE 2 model of M/s Applied Separations, Allentown, USA (Fig. 1) was used. It comprises of a modifier pump (Speed MAX P/N 7025), fitted with refrigerated cooling bath to chill the pump head at -2°C. 10g of sample was charged into a 50ml extraction vessel (SS 316). The flow rate of CO_2 (food grade) was maintained constant at 25.0cm^3/**s** for both sub and supercritical processes. The optimized conditions (obtained by several trials) used for subcritical extraction were 28°C, 65 bar, with static and dynamic time of 30 min each. To obtain a eugenol-rich extract, the SC-CO_2 extraction conditions were chosen using a central composite rotatable design (CCRD) using two parameters of extraction. Extraction temperatures (40, 55, 70°C) and extraction pressures (150, 300, 450 bar) were varied at three levels. The experimental domain of two factors of CCRD consisted of 10 runs. The ten experimental runs consisted of 4 factorial points, 4 star points and 2 central points. The duplication of the central point was used to find the experimental error in the study. Three independent extraction runs were conducted for a given set of extracting conditions with three independent batches of bay leaf powders. The extracts obtained were waxy, semi-solid in nature, were gravimetrically weighed and successively stored in amber-colored screw capped glass vials at 4°C (post dilution in minimum amount of food grade ethanol), until further analyses.

The kinetics of the SC-CO_2 extraction process was studied under the optimized conditions of extraction. Extracts were collected at different time intervals (dynamic time) and the yields of eugenol in the extracts were determined by densitometric analyses.

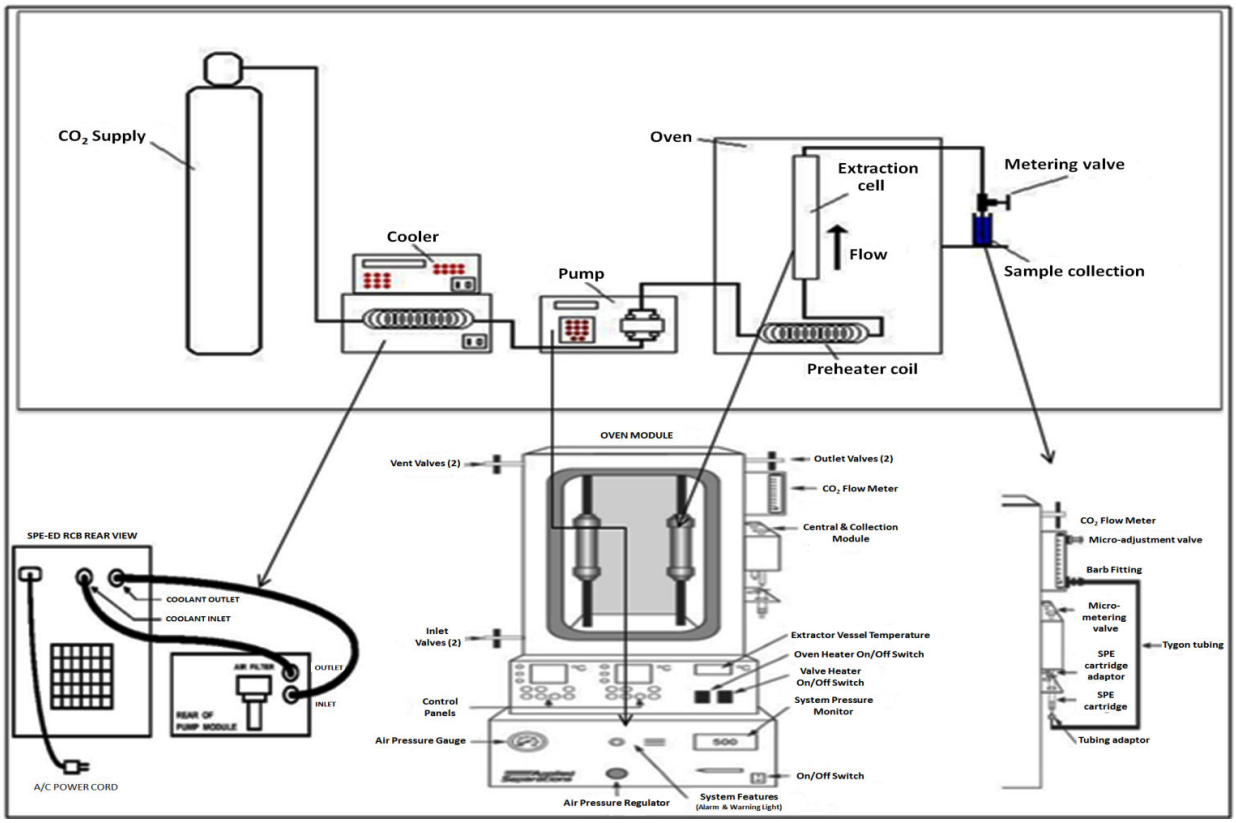

Fig. 1 Experimental unit used for supercritical CO_2 extraction

D. Conventional Methods of Extraction

Hydro distillation of 10g of bay leaf powder was carried out for 5h with 300ml distilled water using conventional steam distillation set up and Clevenger apparatus. Solvent extraction by shake flask method with constant rotatory shaking (190 rpm) was also carried out using same quantity of raw material and 50ml of food grade ethanol at 30°C for 5h. The extracts were then concentrated on a rotary vacuum evaporator (Rotavac system M/s Buchi, Switzerland) at 50 mbar Hg and 50-55°C and finally dried by purging a gentle stream of nitrogen. Extracts were stored in amber colored screw capped glass vials at 4°C for further analyses.

E. Characterization of Bay Leaf Extracts

All extracts were characterized for their phytochemical properties using standard biochemical assays, densitometric and chromatographic techniques.

1. Evaluation of Phytochemical Properties of Bay Leaf Extracts

The following phytochemical assays were performed with the bay leaf extracts. From the respective standard curves, total phenolic compounds were estimated using Folin-Ciocalteu reagent [21] and expressed as g gallic acid equivalent/g dry bay leaf powder and the reducing power as mg BHT equivalent/g of dry bay leaf powder, by the method of Oyaizu [22]. The antioxidant activity was assayed by estimating the radical scavenging activity of DPPH [23] and the anti-inflammatory activity by the *in vitro* nitric oxide (NO) scavenging assay and were expressed as IC_{50} values [24], [25].

2. Densitometric Analyses of Bay Leaf Extracts for Eugenol Content

Densitometric estimation (considering eugenol as the reference standard) for eugenol content in the extracts was performed in accordance to the method described by Bhattacharjee et al. [18], with modifications. 10µl (10 x 10^{-3} cm^3) of all bay leaf extracts, diluted in ethanol were applied in the form of bands, 8 mm wide with 13.6mm spacing between consecutive bands, using a Camag Linomat V (M/s Camag, Switzerland) on Al TLC plates (20cm × 10cm), coated with silica gel 60 (F_{254}). The plates were developed at (23±2)°C in a glass chamber saturated with the mobile phase - toluene: ethyl acetate (93:7). Eugenol showed an R_f value of 0.43 on plate development. The amount of eugenol present in the extracts was determined from the standard curve prepared for pure eugenol at 281nm with Camag HPTLC unit (TLC scanner III).

3. GC-MS Analysis of SC-CO_2Extract of Bay Leaf

Based on the above phytochemical and densitometric analyses, the eugenol-rich SC-CO_2 extract obtained at 55°C and 512 bar having the best combination of phytochemical properties was analyzed by GC-MS for identification of its chemical constituents. A Polaris Q Mass Spectrometer coupled with Trace GC Ultra Gas Chromatography and DB-5 MS fused silica capillary column (30 x 10^3cm × 0.025 cm i.d; 0.25 x 10^{-3}cm film thickness) was employed. The oven module was programmed as follows: it was held isothermally at 85°C for 3min, then increased at the rate of 2°C/min to 200°C with holding time of 1min; then further increased to 250°C at the rate of 3°C/min with holding time of 5 min and finally increased to 300°C at the rate of 10°C/min and held for 15min. Helium was used as the carrier gas at a flow rate of 0.02 cm^3/s. 1µl (1 x $10^{-3}cm^3$) of the sample was injected in split less mode through the injection port held at 280°C. The ionization of the sample was achieved in the EI mode (70 eV) and the acquisition mass range was set in the range of 35 to 350 amu. The chemical compounds in the extracts were identified by computer matching of the chromatogram peak profiles with the NIST (2007) library and with established literature reports [5], [26], [27].

4. Determination of Antimicrobial Activity of SC-CO_2 Extract of Bay Leaf by Microbroth Dilution Method

The antimicrobial potency of the SC-CO_2 extract of bay leaf yielding maximum amount of eugenol and phytochemical properties was determined from the minimum inhibitory concentration (MIC) values against three international strains of microorganisms (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853) in accordance to the method of Chakraborty et al. [28], with little modifications. For MIC determination, Mueller-Hinton broth was used in the broth dilution method. The microwells were fitted with Muller-Hinton broth (100µl) (100 x 10^{-3} cm^3) to which 100µl (100 x 10^{-3} cm^3) extract was added and serially double diluted into eight microwells. 10µl (10 x 10^{-3} cm^3) of microbial culture broth was subsequently added in each well of the 96 well microtiter plate. The microorganisms were incubated in a BOD incubator at 37°C for 24h. The inhibitory effect of the extracts on the growth of the microorganisms was monitored by measuring the optical density at 620nm in microtiter wells at 0h and 24h using a micro plate reader (M/s Micronaut System, Germany).

F. Statistical Analysis of Yield of Bay Leaf Extracts under Different Extraction Conditions

Statistical analysis such as one-way ANOVA has been carried out to study the effect of extraction parameters on the yield of bay leaf extracts. The optimization of yield of the same was conducted by generation of response surfaces followed by their characterization by regression modeling. A p value of 0.05 was used to verify the significance of all tests. All statistical tests of this experiment were conducted using STATISTICA 8.0 software (Statsoft, OK, USA) and MATLAB® Version 7.6.0.324 (R2008a).

III. Results and Discussion

A. Yield of Bay Leaf under Different Conditions of SC-CO_2 Extraction

The yields of extracts (Fig. 2) along with their eugenol content (evaluated densitometrically) obtained under different conditions of SC-CO_2 extraction from powdered bay leaves are shown in Table I. The extracts were waxy and semi-solid in nature, suggesting co-extraction of cuticular waxes. From

the experimental data, it is evident that there is a significant increase in extract yield (p = 0.01) and its corresponding eugenol yield (p = 0.04), with increase in extraction pressure. Although similar effect of pressure on yield of extract was obtained from earlier studies on *Laurus nobilis* [8], [12], [13], no data is available to compare the effect of pressure on yield of eugenol from bay leaves. It is known that the extract yield is mainly determined by the solubility of the essential oil and oleoresin compounds in extracting solvents. Therefore increasing the solvent power of SC-CO_2 with increasing density, leads to higher solubility of these fractions [29]. These justify the usage of high pressure zone for extraction of eugenol-rich fraction from bay leaves.

Temperature has insignificant effect on extract yield (p = 0.56) and also on its eugenol content (p = 0.85). However, it is observed that at low pressure regimes (< 250 bar), there is an increase in extract yield when the temperature is decreased and at high pressure regimes (> 250 bar), there is an increase in extract yield with increase in temperature. As a result, a region of retrograde behavior of solubility of solutes in SC-CO_2 was observed hereby combined effect of pressure and temperature, as is characteristic of SC-CO_2 systems [29].

Fig. 2 SC-CO_2 extracts of bay leaves obtained at different extraction conditions

TABLE I
EXPERIMENTAL YIELD OF SC–CO_2 EXTRACTS OF BAY LEAF AND EUGENOL YIELD ALONG WITH ITS PHYTOCHEMICAL PROPERTIES

Extraction pressure (Bar)	Extraction temperature (°C)	Yield of bay leaf extract (mg/g drybay leaves)	Yield of eugenol(mg/g dry bay leaves)	Total phenolic content (mg gallic acid equivalent/g dry bay leaves)	Reducing power (mg BHT equivalent/g dry bay leaves)	IC_{50} value of DPPH radical scavenging activity(mg/ml)	IC_{50} value of NO radical scavenging activity (mg/ml)
512	55	53.88±0.04	0.72±0.05	1.77±0.04	0.80±0.07	0.20±0.01	1.89±0.01
300	33.78	36.11±0.03	0.54±0.05	1.20±0.04	0.79±0.07	0.32±0.03	2.01±0.02
450	40	66.12±0.04	0.53±0.04	1.05±0.03	0.68±0.06	0.52±0.06	2.99±0.03
300	76.21	65.13±0.03	0.58±0.03	1.02±0.03	0.63±0.04	0.58±0.07	3.01±0.04
450	70	81.12±0.03	0.55±0.02	0.95±0.02	0.61±0.03	0.62±0.02	3.72±0.03
300	55	58.88±0.02	0.55±0.03	0.88±0.02	0.59±0.03	0.69±0.07	3.98±0.06
150	70	42.45±0.01	0.59±0.03	0.46±0.01	0.53±0.02	0.74±0.09	4.04±0.04
300	55	58.78±0.02	0.52±0.03	0.49±0.01	0.48±0.04	0.72±0.05	4.19±0.06
150	40	45.55±0.01	0.47±0.02	0.48±0.01	0.30±0.02	0.86±0.06	4.38±0.04
87	55	4.44±0.01	0.22±0.01	0.20±0.01	0.17±0.01	1.86±0.08	5.67±0.06

[x]Yield of bay leaf extracts, total phenolics, reducing power, IC_{50} of DPPH radical scavenging activity and IC_{50} of NO radical scavenging activity of bay leaf extracts are mean ±SD of three independent extraction runs of three batches of bay leaves.

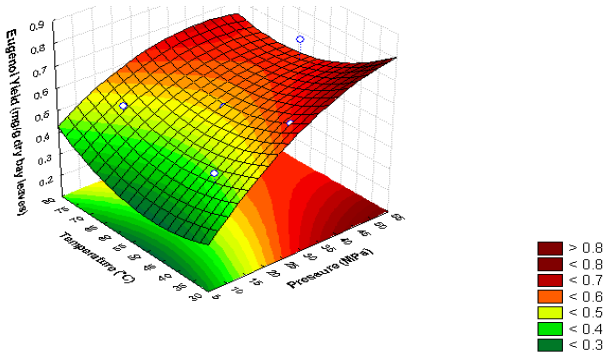

Fig. 3 Response surface indicating yield of eugenol as a function of extraction temperature (40, 55, 70 °C) and pressure (150, 300, 450 bar) at 60 min with flow rate of 25.0 cm^3/sfor 10 g batch size

B. Optimization of SC-CO_2Extraction Parameters to Obtain Maximum Eugenol

1. Generation of Response Curves

The yields of eugenol from bay leaves with varying SC-CO_2 extraction pressure and temperature are shown in Fig. 3.

2. Regression Modeling

Regression modeling was carried out 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

$$Y = B_0 + \sum B_i X_i + \sum B_{ii} X_i^2 + \sum B_{ij} X_i X_j \quad (1)$$

where, Y represents experimental response (yield of eugenol), B_0, B_i, B_{ii}, and B_{ij} are constants and regression co-efficients of the model; and X_i and X_j are independent variables. The

expanded model includes linear, quadratic and cross-product terms as shown below (with intercept):

$$Y = B_0+B_1X_1+B_{11}X_1^2+B_2X_2+B_{22}X_2^2 + B_{12}X_1X_2 \quad (2)$$

$$Y = 0.5375 + 0.1049X_1 – 0.0343X_1^2 – 0.0069X_2 + 0.0376X_2^2 – 0.0250X_1X_2 \quad (3)$$

in which X_1 and X_2represent extraction pressure and temperature respectively and Y is yield of eugenol (mg/g drybay leaves). Equation (3) could explain the effect of independent variables (pressure and temperature) on the yield of eugenol (Y). The correlation coefficient (r) is 0.85. From these moderately high values of correlation coefficients, a statistically significant multiple regression relationship between the independent variables and the responding variables were established.

To check for the adequacy of the above regression model and violations of the basic assumptions of the same, residual analysis has been performed with the experimental data [30].Examination of the residual shows the residual to be 'structureless', i.e., having no obvious pattern, which proves the adequacy of the model [31]. Plot of the observed vs. the predicted values of eugenol yield was obtained. This plot shows a fairly close fit (r = 0.85) of the observed values with the predicted ones (Fig. 4). Thus statistically significant multiple regression relationship between the independent variables and the responding variable could be established.

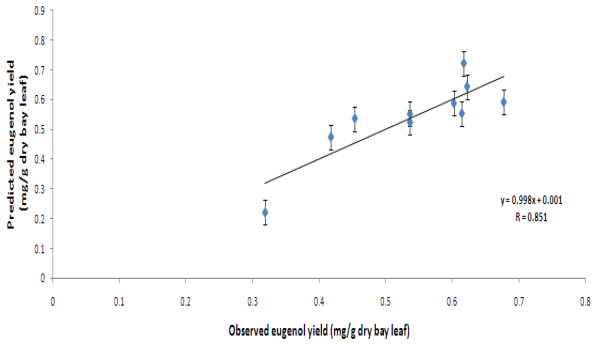

Fig. 4 Observed vs. predicted yield of eugenol in SC-CO_2 extract of bay leaves

3. Analysis of Response Surfaces

The response surfaces have been shown in Fig. 3. From the test statistics for the regression model as discussed above, it is seen that the second order term of extraction, i.e., pressure, had the most significant effect on yield than any other combination of extraction parameters.

4. Optimal Processing Conditions

To determine the optimal processing conditions of SC-CO_2 extraction of eugenol from bay leaves, the optimal values of X_1 and X_2 were determined. The first partial derivatives of the regression equation were conducted with respect to X_1 and $X_2$and set to zero. This was achieved by putting the second-order regression equation in matrix form as described by Montgomery [32] and Ge et al. [33]. The points thus obtained are known as the stationary points: X_{1S}= 501 bar and X_{2S}= 63.02°C. The yield of eugenol (Y_s) obtained at these stationary points was found to be 0.60 mg/g dry bay leaves.

5. Characterizing the Response Surfaces

The response curve was characterized by determining whether the stationary point obtained in the curve is a point of maximum response, minimum response or a saddle point. For this purpose, the regression equation was transformed to the canonical forms and the eigen values were determined in accordance to the method described by Montgomery [32]. Since the eigen values for eugenol yield (0.0002– 0.0002) were of different signs, the optimum point obtained was a saddle point.

6. SC-CO_2 Extraction Conditions for Obtaining Eugenol-Rich Fractions of Bay Leaves

From RSM, it was found that the stationary conditions (X_{2S}= 501 bar and X_{2S}= 63.02°C) obtained for the yield of eugenol is a saddle point. However, the statistically predicted yield of eugenol (0.60 mg/g dry bay leaves) obtained under these conditions was close to our experimental yields. The maximum yield of eugenol-rich fraction (0.72 mg/g dry bay leaves) was obtained at 55°C, 512 bar from our experimental data. ANOVA study revealed that the extraction pressure is the most active and important processing parameter in SC-CO_2 extraction process and in our investigation, the yield of eugenol from bay leaves increased significantly (p = 0.05) with it. However, an extraction pressure greater than 512 bar was not experimented owing to safety constraints of the laboratory scale unit employed in this study. Eugenol extracted at low pressure region (70°C and 150 bar) was significantly lower (p = 0.02) than at high pressure region (55°C and 512 bar). Also the phytochemical properties such as total phenol (p = 0.05), reducing power (p = 0.00), antioxidant property (p = 0.00) and anti-inflammatory property (p = 0.00) were significantly higher in the extract at high pressure region than at low pressure region. Besides, at high pressure region, a significant amount of chlorophyll (determined by method described by Lichtenthaler and Wellburn [34]) was co-extracted whose derivatives are known to exhibit health-promoting activities such as wound healing and anti-inflammatory properties and have a promising role in decreasing risk of colorectal cancer [35]-[37] which was not obtained at low pressure region (70°C and 150 bar). Therefore, within the pressure zone investigated, the experimental conditions of 55°C and 512 bar are considered as the conditions for maximum yield of eugenol from bay leaves. From the kinetic study shown in Fig. 5, it is evident that within 20 min of dynamic time, almost 90% of eugenol was extracted from the leaf matrix and hence the dynamic time of 30 min has been kept constant to obtain maximum yield of eugenol.

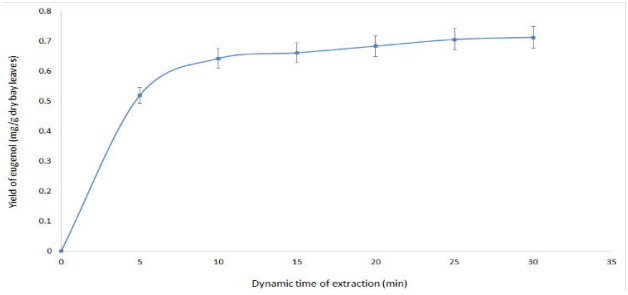

Fig. 5 Yields of eugenol with varying dynamic time of extraction at 55°C and 512 bar

C. Phytochemical Properties of the SC-CO_2Extracts

The total phenolic content, reducing power, antioxidant and anti-inflammatory properties of the SC-CO_2 bay leaf extracts obtained under different extraction conditions are presented in Table I. It is observed that there is a significant increase in the total phenolic content ($p = 0.025$) and reducing power ($p = 0.02$) with increasing pressure; however, there is no significant change for phenolic content ($p = 0.43$) and reducing power ($p = 0.86$) with change in temperature. Similar trends with temperature and pressure were observed for antioxidant and anti-inflammatory properties of the extracts. With change in pressure, there are significant changes in antioxidant ($p = 0.05$) and anti-inflammatory activities ($p = 0.04$); however, no significant changes in antioxidant ($p = 0.75$) and anti-inflammatory activities ($p = 0.47$) were observed with change in temperature. The best combination of phytochemical properties in the extract obtained by SC-CO_2 was at conditions of 55°C, 512 bar attesting to eugenol-rich content of the bay extract obtained under these conditions.

D. Yield of Bay Leaf Obtained by Different Extraction Procedures

The yields of extracts and eugenol obtained by SC-CO_2 extraction under optimized conditions was compared with those obtained by liquid CO_2, subcritical CO_2, steam distillation and solvent extraction (Figs. 6 (a), (b)). It was found that the SC-CO_2 extraction gave the maximum yield of eugenol from bay leaf powder, *vis-à-vis* other extraction procedures, followed by solvent extraction. A steady increase of eugenol yield was observed as we proceeded from liquid to SC-CO_2 extraction. Poor yields of eugenol were obtained by hydro-distillation, liquid CO_2 and by subcritical CO_2, possibly owing to poor penetrability of these solvents into the leaf matrices; while the highest yield of eugenol was obtained by SC-CO_2 extraction owing to enhanced penetrating capacity, higher solvating power and selectivity of SC-CO_2 for this moderately polar compound. Although yield of eugenol was appreciable in solvent extraction, the extract would have poor acceptance owing to toxic solvent residues. In order to further enhance the recovery of eugenol, mucilage from the bay leaves was removed according to the method of Singh et al. [38], prior to SC-CO_2 extraction. However, no significant increase in eugenol yield was obtained on mucilage removal.

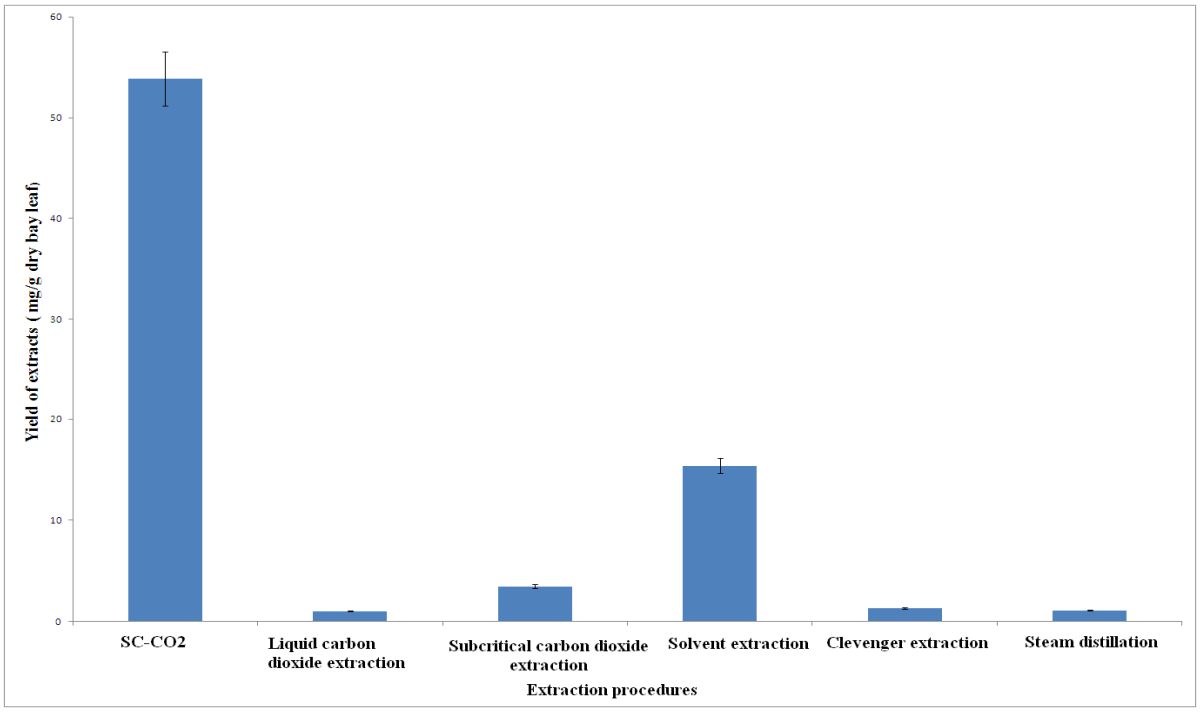

Fig. 6 (a) Comparison of extract yields obtained with different extraction procedures

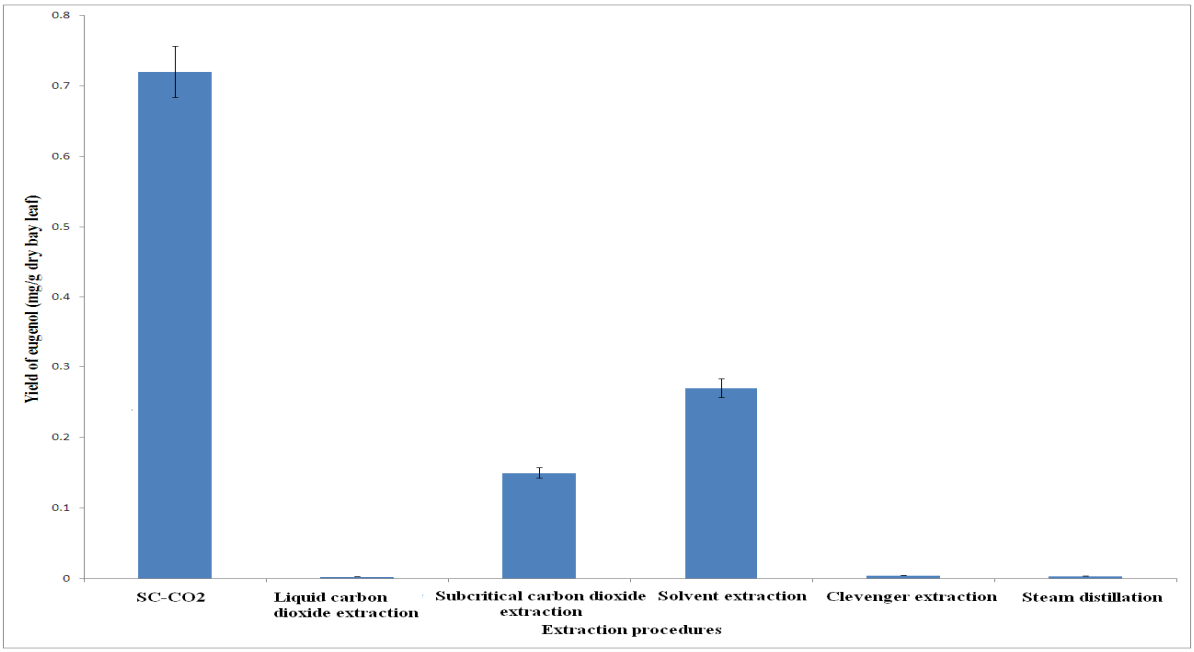

Fig. 6 (b) Comparison of yields of eugenol obtained with different extraction procedures

E. Phytochemical Properties of the Bay Leaf Extracts Obtained by Different Procedures

Phytochemical analyses of bay leaf extracts obtained by different procedures are presented in Table II. From the ANOVA study, it was found that there were significant differences ($p = 0.00$) between the phytochemical properties of the extracts obtained by different procedures. The maximum total phenol content (1.77 mg gallic acid equivalent/g dry bay leaf powder), reducing power (0.80 mg BHT equivalent/g dry bay leaf powder), maximum anti-inflammatory (IC_{50} of NO radical scavenging assay is 1.81 mg/ml) and antioxidant (IC_{50} of DPPH radical scavenging assay is 0.20 mg/ml) activities have been exhibited by the SC-CO_2 extracts.

TABLE II
COMPARISON OF PHYTOCHEMICAL ASSAYS WITH DIFFERENT EXTRACTION PROCEDURES

Extraction procedure	Total phenolic content (mg gallic acid equivalent/g dry bay leaves)[x]	Reducing power (mg BHT equivalent/g dry bay leaves)[x]	IC_{50} value of DPPH radical scavenging activity(mg/ml)[x]	IC_{50} value of NO radical scavenging activity (mg/ml)[x]
SC- CO_2*	0.20±0.01	1.77±0.04	0.80±0.07	1.81±0.01
Liquid - CO_2	2.97±0.03	0.03±0.01	0.05±0.01	11.77±0.08
Subcritical	1.98±0.02	0.13±0.02	0.10±0.01	7.98±0.04
Solvent	1.49±0.01	0.34±0.01	0.48±0.02	3.01±0.02
Clevenger	4.81±0.04	0.05±0.01	0.07±0.01	9.78±0.04
Steam	5.11±0.03	0.05±0.01	0.04±0.01	10.29±0.03

[x]Yield of eugenol, total phenolics, reducing power, IC_{50} of DPPH and NO radical scavenging activity of bay leaf extracts are the mean ± SD of three independent extraction runs of three batches of bay leaf powder.
*(Obtained at 512 bar and 55°C).

F. Effect of Eugenol on Phytochemical Properties of the SC-CO_2 Extracts

A significant correlation is observed for total phenol ($r = 0.85$, $p = 0.00$) and reducing power ($r = 0.94$, $p = 0.00$) with eugenol content of the extracts. Similar trends were observed for antioxidant ($r = 0.99$, $p = 0.00$) and anti-inflammatory properties ($r = 0.93$, $p = 0.00$).

G. Obtaining Eugenol-Rich Bay Leaf Extract with Best Combination of Phytochemical Properties

From the above study, it was found that among the bay leaf extracts obtained by different procedures, the SC-CO_2 extract obtained at 55°C and 512 bar has maximum eugenol content with best combination of phytochemical properties (total phenolic content, reducing power, antioxidant activity and anti-inflammatory properties). This established the superiority of SC-CO_2 extraction over other conventional extraction procedures. This extract was further subjected to GC-MS analysis and antimicrobial activity.

H. GC-MS Analysis of the Optimized SC-CO_2Extract

The compounds in the eugenol-rich fraction of SC-CO_2 extract (55°C, 512 bar) of bay leaf were identified by GC-MS analysis (Fig. 7) and have been reported in Table III. It is observed that eugenol is one of the major compounds in the

bay leaf extract; besides β-sitosterol, α- pinene, β- elemene, β-caryophyllene, spathulenol, caryophyllene oxide and cinnamayl acetate, all of which are reported to have nutraceutical properties [14].

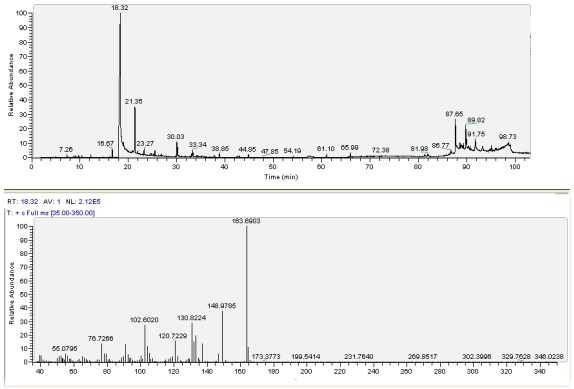

Fig. 7 GC-MS chromatogram of SC-CO_2 extract of bay leaves obtained at 55°C, 512 bar and 60 min extraction time

TABLE III
LIST OF MAJOR COMPOUNDS IDENTIFIED IN GC-MS

Peak. No	R.T. (min)	[M]+ (m/z)	Base Peak (m/z)	Peak Area (AU)	Identified Compounds [x]
1	7.26	136	93	18599	α-pinene
2	16.67	N.A	81	295765	β-elemene
3	18.32	164	163.69	10550974	Eugenol
4	21.35	204	93	1875318	β-caryophyllene
5	30.03	220	43	585813	Spathulenol
6	38.85	204	93	45624	Bicyclogermacrene
7	44.85	176	43	36259	Cinnamyl acetate
8	54.19	N.A	62	15169	NI
9	65.99	220	43	97429	Caryophyllene oxide
10	86.77	N.A	71	240903	NI
11	87.65	486	357	2158860	β-sitosterol
12	98.73	426	43	509307	Lupeol

[x]Identifications were carried out using NIST 2007 and R.P. Adams (2007) [27] , Identification of essential oil components by gas chromatography/mass spectrometry.AU, N.A and NI stand for arbitrary unit, not available and not identified respectively.

I. Microbiological Analysis of the Optimized SC-CO_2Extract

Thus MIC values (Table IV) of bay leaf extracts (55°C, 512 bar) against *E. coli*, *S. aureus* and *P. aeruginosa* were 0.5 mg/ml, 0.50 mg/ml and 0.25 mg/ml respectively. The antimicrobial potency of the extract against these pathogens would be beneficial for use of this extract as a food preservative.

TABLE IV
LIST OF MAJOR COMPOUNDS IDENTIFIED IN GC-MS

Sl.No	Microorganism	MIC (mg/ml)
1	*Escherichia coli (*ATCC 25922)	0.50
2	*Staphylococcus aureus* (ATCC 25923)	0.50
3	*Pseudomonas aeruginosa* (ATCC 27853)	0.25

IV. CONCLUSIONS

The SC-CO_2 extraction conditions that gave the maximum yield of extract from powdered bay leaves (*Cinnamomum tamala)* were at 70°C, 450 bar and 60min extraction time. However, the extract with maximum content of eugenol and best combination of phytochemical properties was obtained at extracting conditions of 55°C, 512 bar and 60 min extraction time. From the comparative study on phytochemical properties of all extracts obtained by different extraction techniques, it was found that the SC-CO_2 extract had the highest eugenol content along with phenolic content, reducing power, antimicrobial, anti-inflammatory and antioxidant activities. We could successfully employ SC-CO_2 extraction technology in extraction of eugenol-rich extract from bay leaves with appreciable nutraceutical potency. This extract has promising applications in food and pharmaceuticals.

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