Industrial Crops and Products 47 (2013) 78–85

Contents lists available at SciVerse ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop

Supercritical carbon dioxide extraction of eugenol-rich fraction from *Ocimum sanctum* Linn and a comparative evaluation with other extraction techniques: Process optimization and phytochemical characterization

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ARTICLE INFO

Article history:
Received 9 October 2012
Received in revised form 1 February 2013
Accepted 27 February 2013

Keywords:
Ocimum sanctum
Supercritical carbon dioxide extraction
Eugenol

ABSTRACT

Ocimum sanctum Linn., commonly known as Tulsi in India, is pharmacologically important owing to its active constituents, chiefly eugenol. In this work, supercritical carbon dioxide (SC-CO_2) extraction has been performed with Krishna tulsi to maximize the yield of eugenol in the extracts and comparatively evaluated against conventional extractions. Phytochemical analyses of chlorophyll-lean and chlorophyll-rich SC-CO_2 extracts showed promising results. SC-CO_2 extract with maximum eugenol content (4.631 mg g^{-1} dry tulsi powder) was obtained at 70 °C, 400 bar and 1.5 h extracting time. However under these extraction conditions, appreciable amount of cuticular waxes and chlorophyll co-eluted. Eugenol-enriched (4.141 mg g^{-1} dry tulsi powder) chlorophyll-lean extract was obtained at 50 °C, 100 bar after 1.5 h extracting time. Comparative study of phytochemical properties of extracts obtained by several extraction techniques, established that the SC-CO_2 extract has the best combination of eugenol and phenolic content along with reducing power, anti-inflammatory, antimicrobial and antioxidant activities. The chlorophyll-rich and chlorophyll-lean extracts of SC-CO_2 extracts have comparable phytochemical properties. SC-CO_2 extraction has been successfully employed for extraction of eugenol from Krishna tulsi with appreciable nutraceutical potency. We envisage that both chlorophyll-lean and chlorophyll-rich extracts of the same would have promising applications in food and pharmaceuticals.

1. Introduction

Ocimum sanctum Linn. (family-Lamiaceae), commonly known as tulsi in India, is a medicinal crop characterized by its square stem and specific aroma (Mondal et al., 2009). The plant is annual and thrives well in hot and humid climate. Tulsi has long history of medicinal use and is widely used in food and pharmaceutical industries. It has considerable nutraceutical properties such as antimicrobial (Phadke and Kulkarni, 1989), anti-inflammatory (Singh and Agarwal, 1991) and antioxidative (Kelm et al., 2000; Yanpallewar et al., 2004) attributed to the presence of compounds namely, eugenol, methyl eugenol, β-caryophyllene, humulene and ursolic acid (Satyanarayana and Sen, 2009; Nair et al., 1982). Among the two varieties of *O. sanctum* Linn, Krishna tulsi (dark purple color) and Rama tulsi (green color) are found in India; the former is utilized more due to its enhanced medicinal properties (Nair et al., 1982).

Commercially available tulsi extract is commonly obtained by steam distillation and solvent extraction. Although these processes are relatively inexpensive, they have potential problems of thermal degradation, hydrolysis and are associated with environmental and health hazards, owing to residual solvent in the extracts. Therefore, alternative extraction methodologies such as liquid, subcritical and supercritical carbon dioxide (SC-CO_2) extractions for end use of natural extracts for food and pharmaceutical applications are currently more preferred (Mukhopadhyay, 2000; Wenqiang et al., 2007).

Since eugenol is one of the main therapeutically active constituents of tulsi, the extraction of the same from dried tulsi leaf powder of 'West Bengal origin' (Eastern India) was carried out by conventional procedures (steam distillation and solvent extraction) and by CO_2 extraction technologies (liquid, subcritical and SC-CO_2 extraction). Similar investigations on extraction of eugenol from leaf matrices have been reported. Leal et al. (2006) reported eugenol as one of the major compounds in the SC-CO_2 extract of clove basil (*Ocimum gratissimim*). Marongiu et al. (2005) reported extraction of eugenol-rich fractions from leaves of *Pimenta dioica* using SC-CO_2; whereas, Ivanovic et al. (2010) obtained the same from bay leaves. Fractional separation model was suggested for extraction

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http://dx.doi.org/10.1016/j.indcrop.2013.02.030

of pure essential oil from herbaceous materials (Reverchon, 1992; Reverchon et al., 1993; Reverchon and Osseo, 1994).

In our investigation, dried tulsi powder has been subjected to liquid, subcritical and (SC-CO_2) extraction technologies and the extracts obtained have been comparatively evaluated for eugenol and phenolic content and also for their phytochemical properties such as reducing power, antimicrobial, anti-inflammatory and antioxidant activities; vis-à-vis the extracts obtained by conventional steam distillation and solvent extraction. The SC-CO_2 extraction processes were designed to obtain eugenol-rich extracts with and without chlorophyll for potential use of these as nutraceuticals in food and pharmaceuticals.

2. Materials and methods

2.1. Materials

Authenticated dried powder of Krishna tulsi variety of *O. sanctum* Linn was procured from medicinal plant garden of Ramkrishna Mission Ashrama, Narendrapur, Kolkata (West Bengal), India. The soil in the experimental site was sandy loam in texture with pH ~ 7–7.5 and is subjected to organic cultivation under tropical-temperate climate (30–35 °C, 75–85% RH). Speciality chemicals such as eugenol (99% pure), 1,1-diphenly-2-picrylhydrazyl (DPPH), sodium nitroprusside, Griess reagent and gallic acid were procured from M/s Sigma, India; and Folinciocalteu's phenol reagent (FCR), chloroform, methanol, dichloromethane, *n*-hexane, sodium carbonate, aluminum chloride, potassium acetate and sodium sulfate were procured from M/s E-Merck (India). All chemicals, solvents and buffers used in this work were of AR grade.

2.2. Characterization of tulsi powder

The measurements of mean particle diameter of tulsi powder have been conducted by sieve analysis method by screening the powder samples through a set of standard sieves (5, 10, 14, 20, 24 and 44 Tyler meshes) on a sieve shaker and noting the particle size distributions (Bhattacharjee et al., 2012). The mean particle diameter (d_p) of tulsi powder was determined to be 0.4 mm. The moisture content of the tulsi powder, determined by AOAC method 930.15 (AOAC, 1990) was found to be 10% on dry weight basis.

2.3. Carbon dioxide extraction of tulsi powder

The extraction of dried tulsi powder by liquid CO_2 was carried out in accordance to the method described by McKenzie et al. (2004) with modifications. 20 g of ground tulsi 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, 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. 20 g dried tulsi powder was charged into a 50 ml extraction vessel (SS 316). The flow rate of CO_2 (food grade) was maintained constant at 2.5 l min^{-1} for both sub and supercritical processes. The optimized conditions used for subcritical extraction were 28 °C, 65 bar with static and dynamic time of 60 min and 30 min respectively. For SC-CO_2 extraction, extraction temperature (50 °C and 70 °C) and static time (1 and 2 h) were varied at 2 levels whereas extraction pressure (100 bar, 250 bar, 400 bar) was varied at 3 levels. Liquid CO_2 was compressed to desired pressure and then continuously pumped into the extractor. A dynamic time of 30 min was kept constant for all the trials due to its insignificant effect on yield (concluded from preliminary trials). The extracts obtained were waxy semi-solid in nature, were gravimetrically weighed and stored in amber colored screw capped glass vials at 4 °C (post dilution with minimum amount of food grade ethanol) until further analyses. All experiments were conducted in triplicate for each extraction mode.

2.4. Conventional methods of extraction

In this work, hydro distillation was carried out for 5 h with 300 ml distilled water and 20 g of dried tulsi powder using conventional steam distillation apparatus and Clevenger apparatus. Solvent extraction by shake flask method was also carried out on 20 g dried tulsi powder using 50 ml of food grade ethanol at 30 °C for 3 h, with constant rotatory shaking (190 rpm). The extracts were concentrated on a rotary vacuum evaporator (Rotavac system M/s Buchi, Switzerland) at 50–55 °C and 50 mbar Hg and finally by purging a gentle stream of nitrogen. Extracts were stored in amber colored screw capped glass vials at 4 °C until further analyses.

2.5. Characterization of tulsi extracts

The extracts were characterized for their phytochemical constituents using standard biochemical assays and by densitometric and chromatographic techniques. Besides, anti-microbial potency of the extracts was also evaluated.

2.5.1. Densitometric analyses of tulsi extracts for eugenol content

Characterization of the tulsi extracts was carried out by densitometric method (considering eugenol as the reference standard) in accordance to the method described by Bhattacharjee et al. (2012), with modifications. 15 μl of the diluted tulsi extracts in ethanol were spotted on Al plates (200 mm × 100 mm) coated with silica gel 60 (F_{254}) in the form of bands, 8 mm wide and with 12.3 mm spacing between consecutive bands using a Camag Linomat V (M/s Camag, Switzerland). The plates were developed at (23 ± 2) °C in a glass chamber containing toluene: ethyl acetate (93:7) as the mobile phase in which eugenol showed an R_f value of 0.43. Densitometric studies with Camag HPTLC unit (TLC scanner III) were performed at 281 nm and the amount of eugenol present in the extracts was determined from the standard curve prepared for pure eugenol. Besides eugenol, bands corresponding to chlorophyll and other phytochemicals were also identified by matching with reported R_f values in literature (Anandjiwala et al., 2006).

2.5.2. GC–MS analysis of tulsi extracts

Based on the densitometric analysis, two eugenol-rich SC-CO_2 extracts (chlorophyll-lean and chlorophyll-rich) were selected and analyzed by GC–MS for tentative identification of the compounds therein. A Polaris Q Mass Spectrometer coupled with Trace GC Ultra gas chromatography having DB-5 MS fused silica capillary column (30 m × 0.25 mm i.d.; 0.25 μm film thickness) was used. The oven temperature was programmed as follows: held isothermally at 85 °C for 3 min, then increased to 200 °C at the rate of 2 °C/min and held for 1 min. It was then increased to 250 °C at the rate of 3 °C/min with holding time of 5 min and then increased finally to 300 °C at the rate of 10 °C/min and held for 15 min. The carrier gas was He at a flow rate of 1 ml/min. The injection port temperature was 280 °C and split less mode was selected. The injected volume of extract was 1 μl. The ionization of the sample was performed in the EI mode (70 eV) and the acquisition mass range was set at 35–350amu. Identification of components of the extract was based on its computer matching with the NIST (2007) library and literature reports (Vani et al., 2009; Khan et al., 2010; Devendran and Balasubramanian, 2011).

2.5.3. Evaluation of phytochemical properties of tulsi extracts

Total phenolic compounds of the tulsi extracts was estimated using Folin–Ciocalteu reagent (Spanos and Wrolstad, 1990) as