Enrichment of Bio-Active Phthalides in Celery Seed Oil

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Abstract: *Objective*: To develop an efficient process to obtain a phthalide-enriched nutraceutical fraction from celery seed oil.

Methods: Three approaches *viz.*, fractional distillation, column chromatography and solvent-solvent partition, have been used.

Results: Fractional distillation of celery seed oil (13.7g) afforded i) a limonene-rich fraction (7.6g, 97% purity), ii) a fraction containing β-selinene (2.8 g, 90 % purity) and iii) one containing phthalides (2.9 g, 90 % purity). Solvent-solvent partition of celery seed oil gave limonene (87%) and a fraction containing phthalides (49%), which on further fractionation afforded a phthalide-enriched fraction (90%). By conventional silica gel column chromatography, a product rich in phthalides (53-74%) could be obtained.

Conclusion: Fractional distillation is shown to be a viable method to process celery seed oil into a phthalide-enriched product with high nutraceutical potential.

Keywords: Celery seed oil, enrichment, fractional distillation, phthalides, GC-MS.

INTRODUCTION

Celery (Apium graveolens L.) a herb (Figure **1A**), grown as a biennial or as an annual, is cultivated as a popular vegetable, for the green and blanched leaf stalks (Figure **1B**) and to a limited extent for the edible thickened roots and crowns. Celery seed (Figure **1C**) is a commercially important spice. Celery seeds contain about 2% volatile oil and 15% fixed oil. The major constituents of celery seed oil are (+)-limonene (> 60%), β-selinene (15-18 %) and phthalides (1-3%). The phthalides are reported to be 3-*n*-butyl phthalide, sedanenolide and sedanolide, which are responsible for the characteristic odor of celery [1]. As a medicinal plant, celery has been used as aphrodisiac, antispasmodic, carminative, diuretic, laxative, sedative and tonic. Celery seed oil is extracted by steam distillation and also an enzymatic approach has been reported for the same [2]. Recovery and identification of alcohols and carbonyl compounds in celery seed oil has been reported earlier [3,4]. Preparations of celery are also used for blood purification, for regulating bowel movements, glandular stimulation and as cure for gallstones and kidney stones [5]. Celery seed extracts have been also shown to possess anti-inflammatory properties. The phthalides from celery are the most significant bio-active compounds exhibiting many health benefits like protection against cancer, high blood pressure and cholesterol. Sedanolide has been reported to be active in the reduction of tumours in laboratory animals. Antioxidant, cyclooxygenase and topo-isomerase inhibitory activities have been shown to be associated with these compounds isolated from seeds of *Apium graveolens* Linn [6].

3-*n*-Butyl phthalide and sedanolide isolated from celery seed oil has been shown to induce the detoxifying enzyme glutathione-S-transferase (GST) in the tissues of female mice. After treatment with 3-*n*-butyl phthalide and sedanolide, the tumor incidence was reduced from 68% to 30% and 11%, respectively. About 67% and 83% reduction in tumor multiplicity was also observed with 3-*n*-butyl phthalide and sedanolide, indicating that 3-*n*-butyl phthalide and sedanolide were both active in tumor inhibition and GST assays, suggesting a correlation between the inhibitory activity and the GST-inducing ability [7]. This data suggests that phthalides, as a class of natural bioactive products occurring in edible umbelliferous plants, may also serve as effective chemo-preventive agents.

Specific herbal preparations containing phytochemicals from ginger, cayenne pepper, turmeric, yucca, devil's claw, nettle leaf, alfalfa and celery seeds have been used to treat prophylaxis and in the therapy of joint and connective tissue disorders in vertebrates [8]. Extracts of celery seed have been evaluated for the treatment and prevention of inflammation and gastrointestinal irritation [9]. *n*-Butyl phthalide the major flavour-impact compound of celery volatile oil, possesses the above mentioned health benefits. In view of these health benefits of the phthalides of celery, and also the demand for newer sources of neutraceuticals, phthalide - enriched fraction from

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A B

C

Figure 1: A: Celery Plant. Family: Apiaceae; Genus: *Apium;* Species: A. *graveolens;* Order: Apiales. **B**: Celery stalk. **C**: Celery Seeds.

celery oil would be in high demand and useful for the treatment of hypertension and heart ailments. There are very few reports on the separation of phthalides [10,11]. In the present study, several physical methods were explored to develop a process for the preparation of phthalide-enriched nutraceutical fraction from celery volatile oil.

MATERIALS AND METHODS

Sample

Celery seeds were purchased from local market, Punjab, India and solvents used were of Analytical grade from Merck, India.

Methods

Celery Seed Oil

Celery seeds (250 g) were powdered to pass through BIS 30 mesh sieve and the powder packed into a glass column. Latter was attached to a condenser and steam was passed through the bottom of the column. Steam distillation was carried out for 3 hrs to collect celery volatile oil. Oil collected (5 ml) was dried over anhydrous sodium sulfate and used for the phthalide enrichment studies.

Three approaches have been explored for the enrichment of phthalides in celery seed oil *viz*., (i)

solvent-solvent partition (ii) column chromatography and (iii) fractional distillation

Solvent–Solvent Partition

Celery seed volatile oil obtained by steam distillation, containing limonene (70-80%), β-selinene (15%) and phthalides was subjected to solvent partition using aqueous 65% ethanol in a separating funnel. Two layers were obtained, the upper layer containing limonene and selinene (A), and the lower layer containing phthalides (B). The phthalide layer was subjected to repeated extractions (3 times) using ethyl acetate. The ethyl acetate layer was separated and distilled under reduced pressure (10 mm) which was further subjected to fractional distillation under reduced pressure (1-0.1mm Hg) to obtain further enrichment of phthalide.

Column Chromatography

In this alternate approach tried for the enrichment of phthalides from celery seed volatile oil, silica gel (25 g) was packed in a glass column (18 cm x 2.5 cm id.) with bed height of 10 cm. Five gram of celery oil was loaded on to the column and eluted with hexane. In the beginning, two fractions of 50 ml each were collected. The column was subsequently eluted with varying proportions of ethyl acetate and hexane (2-20% ethyl acetate in hexane) and finally eluted with acetone. Totally 8 fractions were collected. The fractions were freed of solvent and the weight of the residue recorded. The fractions were dissolved in acetone and analyzed by GC [12, 13].

Fractional Distillation of Celery Seed Oil

Celery seed oil (13.7 g) was taken in a round bottom flask with a vigruex column attached, distilled on an oil bath at 5 mm Hg pressure and fractions were collected. First fraction was collected at 59-60° C. The residue (5.4 g) was transferred to a smaller flask and subjected to vacuum distillation at 0.09 mm Hg pressure to collect a second fraction at 78-80° C and third fraction at 108-115° C. Fractions were analyzed by GC, GC-MS [2] and NMR.

Analysis of Fractions by GC and NMR

Shimadzu 15-A Gas Chromatograph with Column-SE-52% on Chromosorb B (10 ft length, 1/8“internal diameter) flame ionization detector, with a temp. programme of initial temperature of 75°C raised to 180°C at the rate of 5°C per min, raised to 200°C at the rate of 2° C per min with injector port temp. 150°C, Detector port temp. 210°C, Carrier gas flow 30 ml/min. These fractions (0.05 ml) were diluted in acetone (1 ml) and 1 µl was injected on GC. Pure fractions/

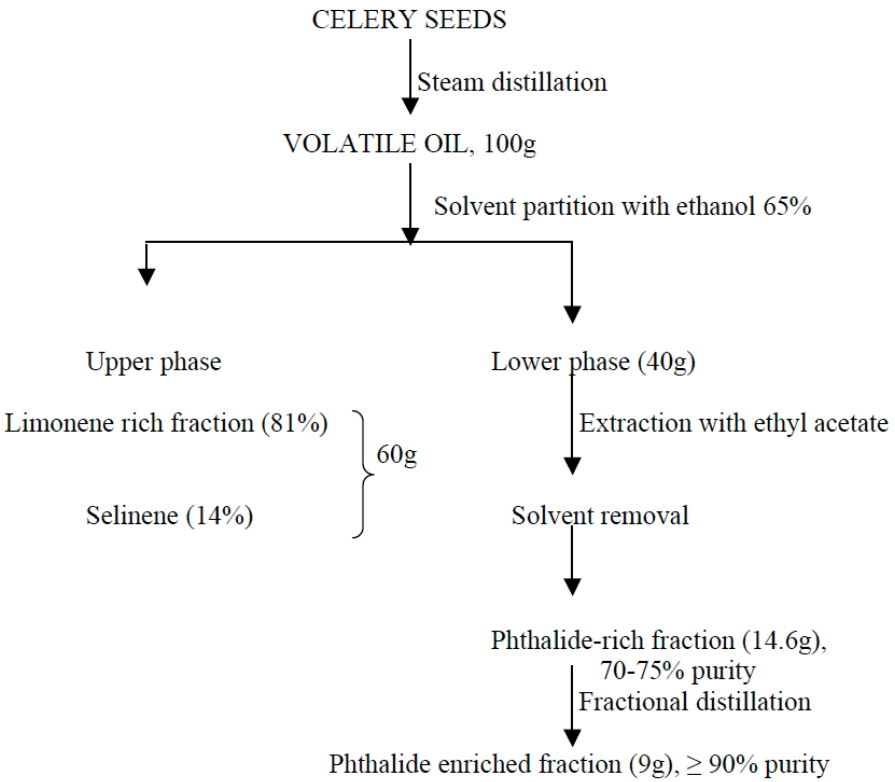


Figure 2: Flow diagram for the preparation of phthalide enriched fraction by solvent-solvent partition.

Table 1: Results of Column Chromatography of Celery Oil Components on Silica Gel

Fraction No.	Eluting solvent	Wt. of the fraction(g)	Limonene (%)	β-Selinene (%)	n-Butyl phthalide (%)	Sedanolide (%)	Sedanenolide (%)
1	Hexane	1.5	81	15.8	-	-	-
2	Hexane	1.5	82	15.2	-	-	-
3	2% ethyl acetate in hexane	0.08	0.8	48.0	-	-	-
4	10%ethyl acetate in hexane	0.3		30.0	-	-	-
5	10% ethyl acetate in hexane	0.14	-	-	74.1	-	-
6	20% ethyl acetate in hexane	0.14	-	-	74.7	5.0	traces
7	20% ethyl acetate in hexane	0.12	-	-	71.4	3.0	traces
8	Acetone	1.2	-	-	90	5.0	traces

compounds (2 mg each) were dissolved in $CDCl_3$ (0.6 ml). ^1H NMR spectra were then recorded on a Bruker Avance AQS, 500 MHz NMR spectrometer [1].

RESULTS AND DISCUSSION

Solvent-Solvent Partition

In one single step by partition of oil (100g) with aqueous alcohol, 60g of the hydrocarbon fraction of the oil (limonene and selinene) could be separated to get a phthalide enriched fraction (40g). The phthalide containing fraction on further extraction with ethyl acetate followed by fractional distillation under reduced pressure of 10 mm Hg afforded phthalide fraction of 75% purity which on further distillation under high vacuum of 0.1 mm Hg afforded an enriched fraction of phthalide (9g) of 90% purity (Figure **2**).

Column Chromatography

By column fractionation of celery seed oil, a phthalide-rich fraction was obtained. The composition of each fraction and yield are given in Table **1**. The first two fractions from hexane showed higher amounts of limonene (81%) and β-selinene (15%), while the fractions 3 and 4 were rich in β-selinene. Fractions 5 & 6 with higher concentrations of ethyl acetate eluted higher concentration of phthalide and traces of limonene. Fraction 8 with acetone showed the presence of phtahlides (90%), which could be a pure

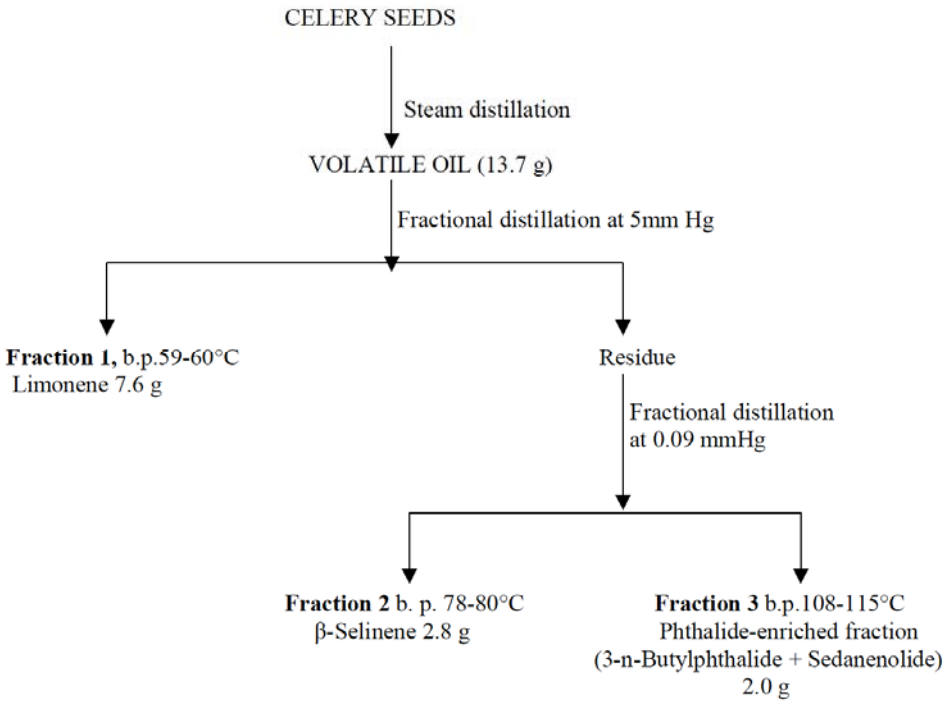


Figure 3: Flow diagram for the preparation of phthalide-enriched fraction by Fractional distillation.

fraction of *n*-butyl phthalide. By adopting this method a phthalide - rich fraction could be obtained from celery seed oil.

Fractional Distillation

In our earlier study, selective collection of volatile oil at different intervals of time of steam distillation (30 min. once) was found to yield fractions with successive enrichment of mono terpenic hydrocarbon, sesquiterpenic hydroterpenic hydrocarbons and phthalides [12]. However, this methodology would not afford very pure phthalide rich fraction. In the present study, conditions were optimized for fractionation of the chemical constituents of celery seed oil. It was observed that by suitable selection of reduced pressures, the constituent terpenes (C_{10} and C_{15}) and phthalides could be effectively separated. The results are presented in Figure **3** wherein celery seed oil (13.7 g) yielded fraction 1 (7.6 g), fraction 2 (2.8 g) and fraction 3 (2 g).

GC Analysis

GC profiles of the 3 fractions obtained by fractional distillation are given in Figure **4**. Fraction 1 (Figure **4A**) had a major peak with a retention time of 4.36 min; Fraction 2 (Figure **4B**) contained a major constituent with a retention time of 14.9 min; 3rd Fraction was mainly a mixture of two compounds (Figure **4C**) with retention times of 18.33 and 19.97 min respectively.

Characterization of Compounds in Fractions I, 2 and 3

Mass spectra of the major compound in Fraction 1 (Figure **5A**) exhibited a molecular ion at 136 with 40 % relative abundance and the base peak was at 68. The latter arises from the retro-Diels-Alder type

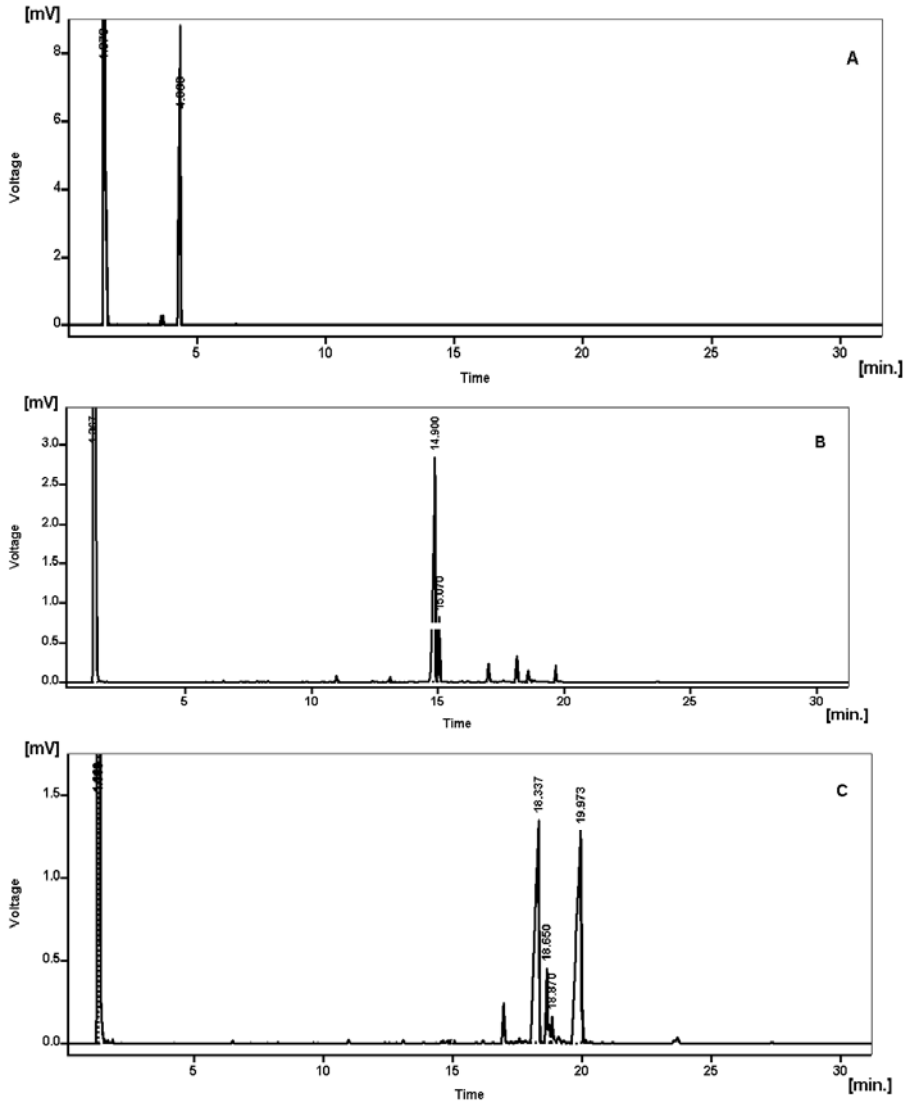


Figure 4: Gas Chromatograph of compounds in fractions 1, 2, 3.

fragmentation from a 1-*p*-menthene skeleton. The fragmentation pattern and the mass spectral comparison with standard spectra indicated the compound as limonene. NMR of this compound (Table **2**) showed an olefinic proton as singlet at 5.42 and two olefinic protons as a singlet at 4.74. The spectrum contained two singlets at 1.68 and 1.76 corresponding to two methyl group substituents on the olefinic bonds. The spectrum also contained multiplets accounting for seven protons of the cyclohexene ring. Mass spectral data along with the ^{1}H NMR data clearly showed it to be limonene.

Fraction 2, showed a major peak at 14.90 min (Figure **4B**). The mass spectral analysis indicated a molecular weight of 204 suggesting it to be sesquiterpenic hydrocarbon. It had a base peak at 93 (Figure **5B**). The fragmentation pattern was representative of a sesquiterpene hydrocarbon and library search results showed it to be β-selinene. NMR of this compound showed two olefinic protons as doublets at 4.74 and 4.50 with J value of 1.5 Hz and two more olefinic protons as double doublets at 4.72 with J values of 1.5 and 5 Hz (Table **2**). The spectrum showed two singlets, one at 1.77 corresponding to a methyl group on an olefinic bond and another at 0.74 for a methyl substituent on a tertiary carbon. The spectrum also contained multiplets accounting for fourteen protons of the saturated naphthalene structural component of the sesquiterpene structure. Mass spectral data along with the ^{1}H NMR data clearly showed it to be β-selinene.

Fraction 3 showed two major peaks with retention times of 18.33 and 19.97 min. (Figure **4C**). The compounds had molecular ion peaks at 190 and 192 respectively. The base peaks in these compounds were at 133 and 107 respectively (Figures **5C** & **5D**). The mass spectral data agree well with those reported for the phthalides in celery [11] (Uhlig, 1987). NMR spectrum contained a distinct set of aromatic protons containing two doublets at 7.88 and 7.44 with J values of 7.5 Hz and two triplets at 7.67 and 7.52 with J values of

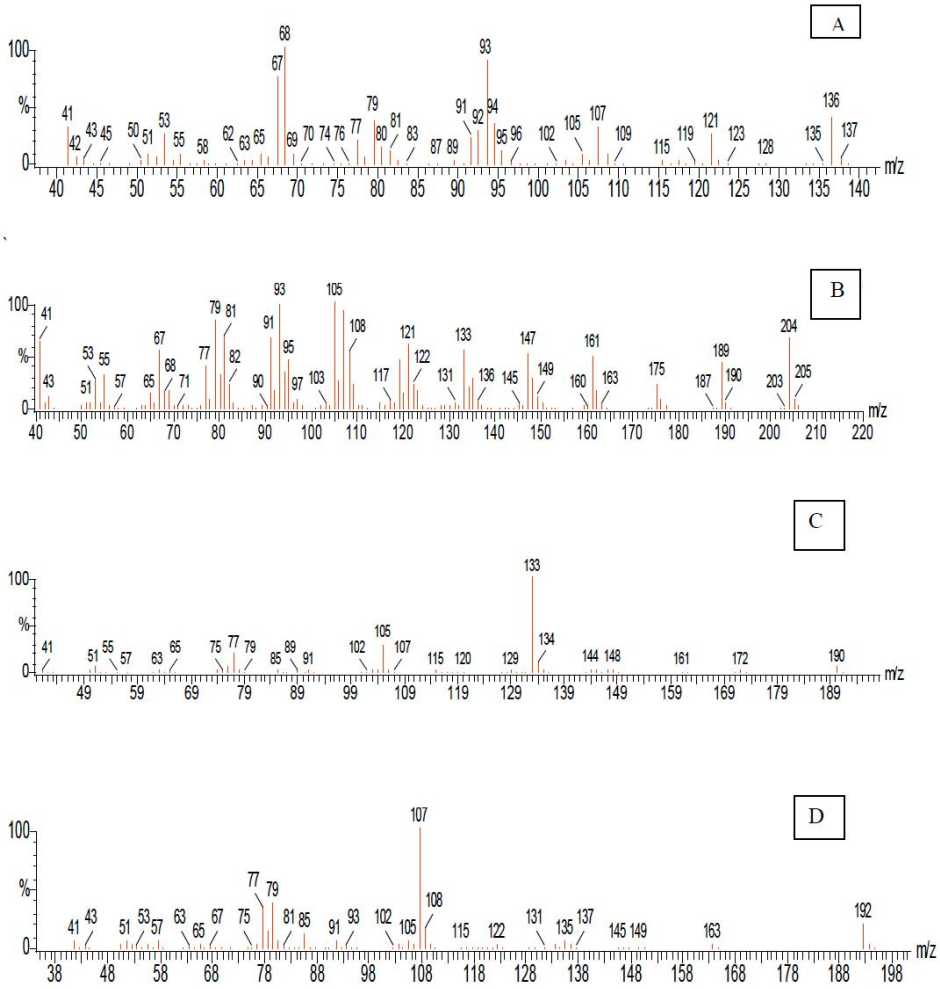
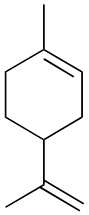
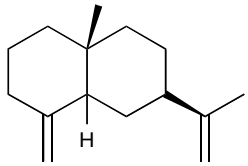
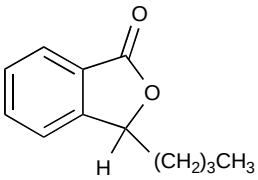
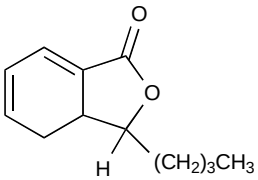


Figure 5: Mass spectra of main constituents of fraction 1 (**A**, Limonene), fraction 2 (**B**, β-Selinene), fraction 3 (**C**, 3- n-butyl phthalide) and **D** (sedanenolide).

Table 2: NMR of Compounds in Celery Oil Fractions

Sl. No.	Compound		^{1}H
Fraction 1	Limonene		5.42 (1H, s), 4.74 (s, 2H), 2.03-2.17 (m, 3H), 1.90-2.03 (m, 2H), 1.79-1.86 (m, 1H), 1.76 (3H, s), 1.68 (3H, s), 1.45-1.55 (m, 1H).
Fraction 2	β–Selinene		4.74(1H,d, j=1.5 Hz), 4.72 (2H,dd, j =1.55Hz), 4.5(1H,d.j=1.542),2.31-2.34(m,1H), 1.98-2.03(m,3H),1.83-1.85(m,1H),1.77(s,3H), 1.43-1.65 (m,9H), 0.74(s,3H)
Fraction 3	n-Butyl Phthalide		7.88 (1 H, d, J =7.5 Hz), 7.67 (1 H , t, J =7.5Hz), 7.52 (1 H, t, J = 7.5Hz), 7.44 (1 H, d, J = 7.5Hz), 1.33-1.48 (m, 6 H), 1.20 (s, 1H), 0.91 (t, 3H, J =7Hz)
Fraction 3	Sedanenolide		6.19 (1 H, d, J = 10Hz), 5.91 (1 H, dd, J = 4Hz), 5.47 (dd, J = 8Hz), 1.33-1.48 (m, 6 H), 1.20 (s, 1H), 0.91 (t, 3H, J = 7Hz)

7.5 Hz (Table **2**). The other characteristic set constituted three olefinic protons one as a doublet at 6.19 with J value of 10 Hz along with two double doublets at 5.91 and 5.47 with J values of 4 and 8 Hz. Apart from these the spectrum contained a multiplet, at 1.33 – 1.48 and a triplet at 0.91 for the methylene and methyl groups of a butyl moiety. The spectrum also contained a singlet at 1.20 for a methine proton. The ^{1}H NMR spectral data clearly showed the compounds to be n-butyl phthalide and sedanenolide.

CONCLUSIONS

Optimization of a simple method to get a phthalide - enriched product from celery seed oil gains importance in view of the various health benefits of phthalides. Three methods *viz*., column chromatography, solvent-solvent partition and fractional distillation were studied and found amenable for obtaining phthalide-enriched product. Fractional distillation affords phthalide–enriched fraction (>95% purity) in 14.6% yield and obviates use of a solvent in contrast to the other two methods. Solvent-solvent partition method has the advantages of low solvent consumption and involves two steps of simple operation to get terpene-rich product and phthalide-rich fraction (45%) compared to column chromatography. Present study describes methodologies to obtain an enriched fraction of phthalides with potential application in the treatment of ailments like hypertension and heart ailments. A phthalide-rich product can become one of the most sought after nutraceuticals of the future.

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