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OCTACOSANOL EXTRACTION, SYNTHESIS METHOD AND SOURCES: A REVIEW

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ABSTRACT

Octacosanol are straight chain aliphatic fatty alcohol which consist of 28-carbon chain, which is basically found in epicuticular region of plant like sugarcane, wheat germ oil, rice bran oil etc. and animal source like krill. Octacosanol is waxy in nature and insoluble in water but sparingly soluble in low molecular weight alkanes, chloroform, ethyl acetate etc. Octacosanol used as a nutritional supplement and functional food. Octacosanol under investigational reported for enhanced stamina endurance, cholesterol lowering effect, Parkinson disease, platelets antiaggregatory properties, amyotrophic lateral sclerosis (ALS, Lou Gehrig's disease), cytoprotective use, and atherosclerosis. Octacosanol extracted and prepared by various methods like Soxhlet extraction, Supercritical fluid extraction and synthetically synthesis.

1.Introduction

Stephen De Felice has coined the term Nutraceutical for the food product or its functional components exhibiting nutrition and pharmaceutical properties both. Nutraceuticals can be functional food ingredients or dietary supplements, obtained from natural sources (mostly plants origin). (Taylor et al., 2003) It is taken by many people at worldwide for getting the health promotion or benefits and disease risk reduction. Infinite numbers of bioactive compounds (individually or collectively) are reported with the expected beneficial effects and it has provided many benefits, depending on mechanisms occurred at varied level with their positive effects. (Rapport & Lockwood, 2000) Few decades people more health conscious but their fast, hectic & modern lifestyle which change diet schedule and diet pattern. They move towards junk food and ready to eat food's system in which they lack of essential and non-essential nutrients, mineral, vitamins etc. such types of health-conscious population shift towards Functional food, dietary supplements and Nutraceuticals products. This is obtained from herbal source, marine sources or synthetically derived. Octacosanol, one of the most abundant alcohols in policosanols, is taken as an alternative to aspirin for patients suffering from gastric irritation due to its cytoprotective effects (Varady et al., 2003).

There are various such products are available in market and many more in pipeline to introduce in health market. They available in three stages like extract form, intermediate formulation and finished dosage forms. All stages have their own merits and demerits. Like herbal products are highly unstable towards light, moisture, oxygen and temperature. Herbal products are less bioavailable due water insoluble property. So intermediate formulation performed on initial extract form to improve solubility, stability and bioavailability in finished products.

Octacosanol is one of the popular functional foods. Octacosanol is being used to prepare various kinds of dietary supplements, health foods, and pharmaceuticals (Janikula, 2002).

Cholesterol is an important component in the body as it is a major component of cell membranes, but high levels can cause hypercholesterolemia and eventually atherosclerosis leading to coronary heart diseases. (Gouni & Berthold, 2002) In various human studies, a daily supplement of 5–20 mg policosanol decreased the low-density lipoprotein (LDL) cholesterol concentration between 19 and 31% and the total cholesterol (TC) concentration between 13 and 23%. Long-term studies have shown that high-density lipoprotein (HDL) cholesterol levels increased in the range of 8–29%. (Francini et al., 2008) A daily dose of 40 mg policosanol seems to be effective in reducing the serum triglycerol concentration. Octacosanol improves oxygen usage by strengthening the heart, supporting low LDL and high HDL levels there by maintaining healthy heart function (Norris et al., 1986). Therefore, octacosanol is an important nutraceutical for the future because of increasing problem of obesity and chronic heart diseases (Snider, 1984). Pure octacosanol has been investigated as a possible treatment for Parkinsonism and amyotrophic lateral sclerosis (Beltz & Doering, 1993) it has also been used by athletes to enhance their performance by enhancing stamina (Irmak et al., 2006).

Policosanol, a mixture of long chain fatty alcohols (C24– C34 alcohols) is obtained from plant waxes and beeswax as a solid waxy substance and insoluble in water but Soluble in organic solvents (Ou et al. 2012). Several times the word "policosanol" is being used for labelling the enriched octacosanol for commercial purposes. Octacosanol is isolated from sugar cane wax consisting of a mixture of 1-octacosanol (60–70%), 1-triacontanol (10–15%), 1-tetracosanol (<2%), 1-hexacosanol (3–10%), 1-heptacosanol (<3%), 1-dotriacontanol (5–10%) and 1-tetratriacontanol (<5%) (Kawanishi et al., 1991). Sugar cane wax (6.85 g) was separated from sugar cane juice filter mud (100 g) and 22.52 g of octacosanol was extracted from 100 g of sugar cane wax (Chen et al., 2006). Octacosanol is also isolated from rice bran wax, but its content was never reported to be more than 15–20% in the policosanol mixture (Vijaya et al., 2013). The solvent extraction processes of policosanol from natural substances result in mixtures of fatty alcohols from which it is possible to obtain a single compound of interest only after expensive purification operations (Cravotto et al., 2010).

2.Various Sources, Extraction and Synthesis Process for Policosanol (Octacosanol)

An efficient synthetic method was developed for the preparation of 1-octacosanol (C_{28}-alcohol) from commercially available lipid-based intermediates namely sebacic acid (decanedioic acid) and stearyl alcohol. The key step in the synthesis is the preparation of tert-butyl dimethyl octacos-10-enyloxy silane from 10-tert-butyl dimethyl silanyloxy decanal and octadecyl triphenyl phosphonium bromide salt employing Witting reaction as per Fig.1.

This product on simultaneous hydrogenation of double bond and deprotection of tert-butyldimethylsilyl protecting group in a Single step on treatment with Pd/C and H_2. In methanol at ambient temperature resulted octacosanol in 95% yield. The products were characterised by IR, ^{1}H NMR, and GC–MS analysis. (Cravotto, 2005) Another synthesis of octacosanol from 10-undecenoic acid (UDA) and stearyl bromide. According to their methodology, conversion of UDA to methyl 10-oxo-decanoate was carried out by two routes,

(i) Dihydroxylation of UDA epoxide followed by oxidation and esterification or
(ii) Esterification of UDA, followed by oxidation using osmium tetra oxide.

Octacosanol was obtained from Wittig product after two steps namely, lithium aluminium hydride reduction of ester group to alcohol and double bond hydrogenation using Pd/H_2 under 30 bar pressure. Both the methods employed either ultra sound and micro-wave irradiation methods or expensive reagents like

osmium tetra oxide, m-chloroperoxybenzoic acid (Shin & Kim, 1994).

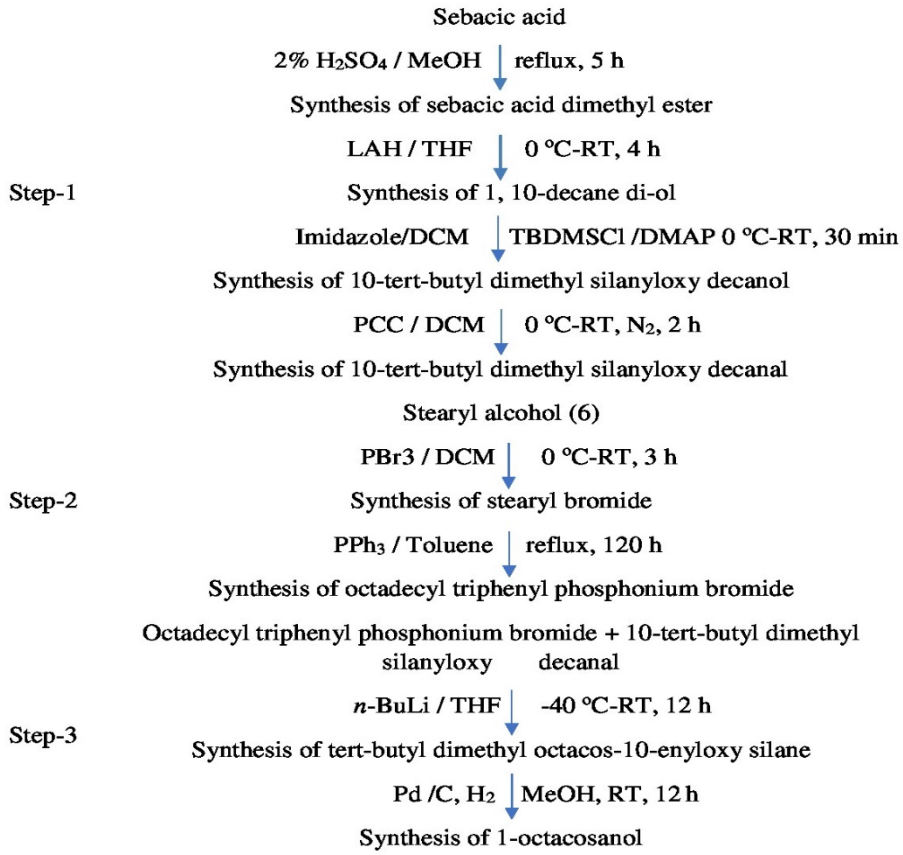

Figure 1. Synthetic route for 1-octacosanol

3.Extraction of Policosanols from Perilla seeds (*Perilla frutescens*)

Perilla seeds (*Perilla frutescens*) are a traditional source of oils produced in Korea, India, China, and other Asian countries. Annual production of perilla seeds in Korea is about 40000 metric tons and expelled perilla oil production is the third largest among edible oils in Korea (Prakash et al., 2006). Perilla seed oil contains considerably high levels of linoleic acid (Hwang et al., 2002a). Despite its nutritional benefits, perilla seeds are largely unknown in Western society. Therefore, the extraction of oils and oil-based products from such plant seeds would provide a new supplement in the world market.

Perilla and sesame seeds (800 g) were washed with tap water and placed on an absorbent towel. Immature seeds were removed by hand. Remaining seeds were dried at room temperature for 24 h. The uncrushed seeds were refluxed with 800 mL of hexane for 30 min in a round-bottom flask (2 L) at 70 °C. The warm mixture of seeds and hexane was filtered through a coffee filter paper to remove seeds. The filtrate was then passed through Whatman No. 2 filter paper to remove impurities and stored at -18 °C for 8 h to precipitate the Policosanols. The filtrate was prefiltered through Whatman No. 42 filter paper. Precipitate on the No. 42 filter paper was vacuum-dried at room temperature and collected as waxy materials (Hwang et al., 2002b).

4.TLC of Policosanols

TLC analyses of Policosanols extracted from the seeds were performed. Waxy material solution in hexane (about 10 μg/10 μL) was

spotted on a 20 x 20 cm, silica gel 60, 250 μm TLC plate. The developing solvent was a mixture of hexane, diethyl ether, and acetic acid (85:15:2, v/v/v). Developed bands were visualized by dipping the plate in 10% cupric sulfate solution containing 8% phosphoric acid for 5 second. Then the TLC plate was dried for 5 minute and kept in an oven at 150 °C until the developed bands were charred. (Hwang et al., 2004**)**

5.Compositional Analysis of Policosanols

Components were determined as per below method, using an HPLC equipped with a 250 mm x 4.6 mm i.d., 5 μm Luna silica column connected with a 4 x 3 mm i.d. guard column. The detector was an all tech Evaporative Light Scattering Detector 800, operated at 40 °C with nitrogen pressure of 3.5 bars. Two Waters 510 HPLC pumps were operated in gradient mode at a flow rate of 1 mL/min. Elution solvent consisted of a gradient of hexane (solvent A) and 0.2% acetic acid in methyl *tert*-butyl ether (solvent B), with the following profile: 0-2 min, 100% A; 3-10 min, 95% A; 14 min, 55% A; 23-26 min, 0% A; and 27-40 min, 100% A. The column and guard column were heated to 38-40 °C using a Waters Column Heater Module. Exposed lines from injection loop to detector connection were maintained at 38-40 °C wrapped with a heating tape. Samples were prepared in hexane (2 μg/20 μL), and 20 μL of each sample was injected for the analysis (Dixit & Khosa, 1971).

6.Compositional Analysis of Policosanols Fractionated from Waxy Materials

The composition of policosanols in the waxy materials was analysed using GC as mention below method. The policosanol fraction (2 mL) collected from HPLC (20 μg of policosanol) was derivatized to trimethylsilyl (TMS) ethers (10 min at 60 °C) using 0.05 mL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide and 0.2 mL of chloroform. A standard solution of alcohols (docosanol, tricosanol, and tetracosanol and hexacosanol, heptacosanol, octacosanol, and triacontanol was prepared in 0.2 mL of chloroform (1-8 μg of each) and derivatized as above for the identification of retention times and the calculation of their response factors. The TMS ether derivatives (2 μL) were injected into a 6980 Series GC equipped with a 30 m x 0.25 mm i.d., 0.25 μm, DB-5 column, flame-ionization detector, and helium as a carrier gas. Injector and detector temperatures were both set at 315 °C. The oven was programmed to start and hold at 150 °C for 1 min before increasing to 210 °C at 20 °C/min, increasing to 310 °C at 4°C/min, holding at 310°C for 1 min, increasing to 315 °C at 25 °C/min, and holding for 5 minute (Morris et al., 2000).

7. Preparation, Purification and Octacosanol from the Leaves of Sabicea grisea

Powder of the dried leaves of *S. grisea* was extracted at room temperature with 90% ethanol. The solution was filtered using a Whatman N° 1 filter paper under suction and concentrated to dryness at 50 °C under reduced pressure. The obtained crude ethanol extract was partitioned between hexane, chloroform and hydro-alcoholic solution (7:3). The hexane fraction was further fractionated on a silica gel column using hexane, containing increasing amounts of ethyl acetate. Fractions with similar thin layer chromatography profiles were pooled. The remaining fractions were subjected to washings with hexane that resulted in the isolation of octacosanol ($C_{28}H_{57}OH$) (45 mg), melting point 81–82 °C. The concentration of octacosanol was 88.23 µg per 1 g dry weight of leaves. Verification of the purity of octacosanol was carried out by gas chromatography. The GC data revealed that a major peak was eluted at 38.79 min. However, minor impurities (especially hexacosanol at 36.08 minute—$C_{26}H_{53}OH$) were also detected as per the GC profile indicating that the compound was about 90% pure (Pollard et al., 1931).

This compound was characterized based on its GC-MS, and 1H and 13C NMR analyses together with available literature. GC-MS analysis of octacosanol was carried out with instrument GC–MS system under the following conditions: DB-5 fused silica column (30 m

length, 0.25 mm i.d., 0.25 mm film thickness); injector temperature, 220 °C; temperature programmed at 60–270 °C at 3 °C/min;

Injection type, splitless (1 μL of a 1:1000 *n*-hexane solution); carrier gas, helium, adjusted to a linear velocity of 32 cm/s (measured at 100 °C); mass spectra, 70 eV, in EI mode; ion source temperature, 180 °C; scan mass range, 25–700 u (Chibnall et al., 1931).

8.The Isolation of n-Octacosanol from Wheat Wax

The isolation of the longer-chain primary alcohol constituents of waxes from the blades of wheat (Triticum vulgare) which air dried in a room at 40°C and powdered material (14 kg.) was extracted with ether and from the material thus obtained (400 g.) the crude wax (62 g.) was prepared (Bleyberg and Ulrich, 1931). The yield was therefore 15-5 % of the ether extract or 0.44 % of the dried wheat. On saponification the wax gave 40 g. of unsaponifiable material, which was a hard-yellow wax, 12.4 g. of crude fatty acids. As a preliminary stage the unsaponifiable wax when treated by the phthalate (Backmann and Clark, 1927). Gave only a primary alcohol M.P. 82-83°C and material was dissolved in warm chloroform which was poured into a dish and left exposed to the air. On evaporation of the solvent a yellow friable powder was obtained, which was then shaken for ten minutes at room temperature with light petroleum. Twelve successive extractions removed all material (10 g.) readily soluble in this solvent, leaving 30 g. of insoluble crude primary alcohol.

9.Constitution of the primary alcohol

The crude primary alcohol was crystallised from carbon disulphide to remove the last traces of paraffin, giving 26.6 g. of white granular crystalline powder, M.P. 82-82.5°. 26 g. were acetylated by boiling gently for 16 hours with acetic anhydride and fused sodium acetate (20 g.). The dark brown solution was poured into water and crushed ice and the mixture stirred for 15 minutes. (Piper et al., 1931) The brown powder was collected, again stirred with iced water and finally dissolved in benzene-methyl alcohol, leaving a tarry residue. From the solution (charcoal) 27.5 g. of crystalline acetate were obtained, M.P. 63-64°. This material was fractionally distilled in vacuum from a Willstatter flask of 100 cc. capacity. The temperature of the metal-bath was about 280°C and several fractions were collected at 185-195°C/0.03 mm. the melting-points of which ranged from 63.5° to 64.5°C. The higher-melting fractions were then collected and redistilled (Ramaswamy et al., 1980).

The principal component of the wax from blades of young wheat is a long chain primary alcohol which has been identified as n-octacosanol (M.P. 83.2-83.4°C) by reduction to n-octacosane (M.P. 61.3-61.5°C) and by oxidation to n-octacosanoic acid (M.P. 90.8-91.1°C). The purity of all three products has been confirmed by X-ray analysis. The wax also contains mixed fatty acids, the composition of which has not yet been determined, and paraffin, M.P. 66°C, which has been shown to be a complex mixture (Cravotto et al., 2004).

10.Extraction of policosanol from rice brain oil by supercritical fluid extraction (SFE)

160 g of freshly obtained rice bran, stabilized using an automated microwave oven at 110°C for 200 seconds and stored at 4°C was extracted using an SFE machine for 3 hours at 60°C, 600 bars pressure, with a carbon dioxide flow rate of 25 g/minute.38 The extracted oil was collected and percentage yield calculated with respect to the rice bran sample weight.

The RBW in the SFE-extracted RBO sample was separated as acetone insoluble (Dunford et al., 2010). Acetone chilled to approximately 4°C was added to 5 mL RBO (1:1, volume/volume [v/v]), and the mixture was centrifuged at 4,000 rpm for 25 minutes. Supernatant oil was decanted carefully, and the insoluble portion was washed with 5 mL chilled acetone and centrifuged. The wax obtained was blown with nitrogen, using a nitrogen generator, and then dried in an oven at 60°C. The dried wax was then weighed and stored at 4°C until further analysed.

Policosanol was extracted from RBW. (Kazuko et al., 1991) About 10 g RBW was

placed into a 200 mL conical flask and hydrolysed with 100 mL 0.2 M NaOH by sonication with a Power sonic 505 ultra sonicator 50 Hz, 350 W, at 60°C for 90 minutes. The hydrolysed mixture was then extracted with an equal volume of petroleum ether, cooled down to 2°C, and then divided into 50 mL centrifuge tubes and centrifuged at 4,000 rpm for 10 minutes. The upper petroleum ether layer and the lower NaOH layer were removed by carefully decanting; the middle yellowish layer (policosanol) was collected and freeze dried.

11.Determination of policosanol content

Policosanol contained in the RBW was determined by gas chromatography mass spectrophotometry (GC-MS). The GC-MS determination of policosanol was done. Policosanol standards docosanol, tetracosanol, hexacosanol, and octacosanol. (Tolloch, 1976). A 5mM concentration mixture of these standards in chloroform was prepared, 0.5 mL of this mixture was derivatized with 0.2 mL N,O-Bis(trimethylsilyl)trifluoroacetamide by incubating at 60°C for 20 minutes, and then the volume was made up to 1 mL by adding more chloroform after cooling to room temperature. RBW policosanol extracts were derivatized the same way. Policosanol standards and RBW policosanol extracts were first injected into the GC-MS machine.

The GC oven temperature was programmed from 150°C to 300°C with a heating rate of 4°C/minute and maintained at this temperature for 15 minutes. Initial flow rate of the carrier gas, helium, was 1.0 mL/minute. Inlet temperature was 300°C. GC-MS parameters were as follows: the MS transfer line temperature was 280°C, the ion source was kept at 230°C, and the MS quadrupole temperature was kept at 150°C. The ionization energy was 70 eV with 2 scans/second and a mass range of 100–1,000 amu. The standards/samples (2 μL) were injected into GC-MS with a 1:10 split ratio.

12.The purification of crude octacosanol extract from rice bran wax by molecular distillation

Octacosanol has been found in many plants, e.g. in leaf, bark and stem waxes of rye grass, apple peel, and wheat germ**.** Rice bran wax (RBW) has been reported to be one of the best sources containing Octacosanol**.** To extract and purify octacosanol from RBW, column chromatography and recrystallization are popular methods (Steve et al., 1963).

Molecular distillation (MD) can avoid using any organic solvents in the purification, even if it can remove any harmful solvent residues in the products, resulting in the generation of a much smaller waste and higher safety (Christensen & Reineccius, 1995 a; Wu & Zhang, 2000 b). Molecular distillation is a special case of short-path distillation in which the distance between evaporating and condensing surfaces is less than the mean free path of the molecules involved in high vacuum**.** (Feng et al., 2002 a; Ridway, 1956 b). This technology is considered as one of the best methods separating and purifying natural product, especially for substances with high-molecular mass, high viscosity and high melting point (Armando et al., 1994 a; Ooi et al., 1994 b). Meanwhile response surface methodology (RSM) is effective for responses that are influenced by many factors and their interactions, which was originally described (Box and Wilson, 1951). Previous research indicated that it is useful for developing, improving and optimizing processes (Atkinson & Donev, 1992 a; San Martin et al., 2003 b). In the this research, MD is used as a main method for the purification of crude octacosanol extract from trans esterified RBW, and its working conditions such as distilling temperature and vacuum degree is optimized by RSM in order to obtain the highest octacosanol content and parallelly to maintain the yield in the purified product as large as possible. The detailed process and the effect of MD conditions on the purification are elaborated by Octacosanol mathematical model; there some of references

have referred to purification of crude octacosanol extract by MD (Feng et al., 2002).

RSM was applied to determine the working conditions of MD for the purification of crude octacosanol extract from RBW. A central composite rotate design (CCRD) was used to investigate effects of two independent variables (purification conditions), distilling temperature and vacuum degree, on dependant variables of the purification.

The Trans-esterification reaction of the RBW was carried out in a round-bottomed flask equipped with a temperature controller and a stainless-steel double-arm blender. The RBW was added to the flask, and heated until it completely melted. An n-butanol solution containing 0.2% KOH was added to the melted RBW with continuous stirring. The above solution was refluxed for 8h. The reaction mixtures were cooled to 0°C, and parallelly washed with distilled water to neutral. The resulting dried solid was extracted with ethanol at 70°C to obtain the crude octacosanol extract.

The MD was used to further purify crude octacosanol extract obtained from Trans esterified RBW. the distilling temperature and vacuum degree were two major factors responsible for the further purification of crude octacosanol extract, while the flow rate of feed, temperature of condensing surface, and rotate rate of scraper were not included as CCRD factors, and process were set at 3ml/min, 90°C, and 50 rpm respectively. The feed was heated to melt, after setting the parameters, feeding valve was turned on, and the degassed feed liquid was introduced subsequently down the evaporating surface and spread with a very thin film by scraper. Heated walls and high vacuum drive the more volatile components had been closely positioned internal condensing surface as the less volatile components continue down the cylinder. The obtained fractions, which separated, collected with individual discharge outlets. The distillates were collected to calculate the yield and determine octacosanol content.

By the reported methods of determine octacosanol content (Kazuko et al. (1991) and Gonzalez et al., 1996), 0.200 g 1, 3, 5-triphenylbenzene (TPB) was dissolved in 100.00ml cyclohexane. The TPB concentration in this solution was 2.000 mg/ml. Calibration curve were obtained by injecting standard solutions with concentrations of octacosanol ranging from 100 μg/ml to 900μg/ml. 0.025 g of sample, which was obtained by trans esterification in conjunction with MD from RBW, was dissolved in 3.0ml cyclohexane under the help of ultrasonic wave at 40°C. Then 1.5ml of 2.000mg/ml TPB solution was added to the above solution as internal standard. The resulting solution was made up to 5.0ml with cyclohexane before being subjected to GLC analysis. GLC was performed with a Hewlett Packard 6890A and a HP-5 (column 30m · 320lm · 0.25lm). The gas flow rates for N_2, H_2 and air were 45, 40, 450ml/min, respectively. The operating temperatures were set as follows: injector, 320°C; detector, 330°C; initial oven temperature 230°C, keeping 6 min., with a ramp rate 10°C/min to 280°C, keeping 20min., then with a ramp rate 20 °C/min to 300°C.

13.Preparation of octacosanol from filter mud by SCFE

Sugarcane is one of the major crops in the world and in China. Which is an ideal source of octacosanol, as its bagasse contains a higher amount of policosanol than sugarcane leaves and other materials, and has a high and stable content of octacosanol (Irmak et al., 2006; Oliaro-Bosso et al., 2009). After cane harvesting and processing, every 1000 kg of cane would produce 33 kg press mud or filter mud (Almazan et al., 1998) that contains 7% of crude wax, in which octacosanol amounts to 81% (Nuissier et al., 2002).

Extractions were performed on a SCFE extractor. 100 g of filter mud was suspended in 500 ml of absolute ethanol in a 1 L stainless extraction vials and extracted with 99.99% CO_2 at a flow rate of 30 L/h. The waxes were collected in a cooled separator at 25°C. The content of octacosanol in the extract was analysed as per prescribed procedure below. Extractions were performed in triplicate. 1kg. of

filter mud was suspended in 8 L of absolute ethanol in a 20 L reactor and refluxed at 80°C, 120 rpm for 4 hrs. After extraction, the processed solution was filtered through 300-mesh filter, the filtrate was cooled to 4°C, after centrifugation the green flocculates obtained, the sediments kept in the open air for 4 hrs to evaporate ethanol and dried in an oven at 60°C. The content of octacosanol in the waxes was determined by GC/MS.

The waxes were subsequently purified by the reported procedures. 10.0 g of the waxes was extracted using 200 mL of acetone in a Soxhlet extractor to remove chlorophyll and fat. The residue was placed in a 250 ml of flat bottom flask containing 100 mL of 95% ethanol and 4 g powdered sodium hydroxide and was refluxed at 80°C for 6 h; the mixture was cooled to 50°C and extracted with 200 mL of petroleum ether three times. The combined petroleum ether phase was cooled to 4°C and then was filtrated using filter paper, the filtrate cake was air-dried.

Policosanols in sugarcane bagasse and filter mud were extracted, for determination of octacosanol in the raw materials (Irmak et al., 2006). Octacosanol in the extracts was analysed (Chen et al., 2007).

1. Octacosanol was analysed on a GC/MS system, equipped with an HP-5 (30 m x 0.25 mm x 0.25 μm) capillary column. The conditions used for the GC measurement were as follows: Oven temperature programmed from 80°C to 320°C, at 10°C/min, and maintained at 320°C for 15 min.
2. Oven temperature programmed from 80°C to 320°C, at 10°C/min, and maintained at 320°C for 15 min.
3. Helium was used as carrier gas at a flow rate of 1.0 mL/min.
4. The inlet temperature was 300°C.

GC/MS operating temperatures were as follows:

1. MS transfer line 280°C, ion source 230°C, and MS quadrupole 150°C.
2. The ionisation energy was 70 eV.
3. The scan range and rate were 50-600 amu and 2 scans/s, respectively.
4. The injection volume was 10 mL.

The calibration curves were obtained by injecting the standard solutions with concentrations ranging from 100 to 900 μg/mL.

14.Octacosanol isolated from Tinospora cordifolia

T. cordifolia, generally known as guduchi, is broadly used in veterinary medicine and Ayurvedic system of medicine for its common tonic, antiperiodic, antispasmodic, anti-inflammatory, antiarthritic, anti-allergic and antidiabetic properties (Singh et al., 2003). Guduchi has been reported to be active against throat cancer in man and it has been reported to be non-toxic in acute toxicity studies in vivo, with almost no side effects (Chauhan, 1995). It has been shown that the polysaccharide fraction from guduchi was found to be very effective in reducing the metastatic potential of B16F-10 melanoma cells (Leyon and Kuttan, 2004a, b). The antiangiogenic and proapoptotic potential of T. cordifolia crude extract or hexane fraction (Leyon and Kuttan, 2004 a, b; Thippeswamy and Salimath, 2007). The pure compound responsible for this activity and its molecular mechanism of action has not been hither to investigate. A long long-chain aliphatic alcohol from T. cordifolia by activity-guided purification and shown that it inhibits tumour-induced angiogenesis in vivo by inhibiting VEGF gene expression. The mechanism of down regulation of VEGF gene expression in is shown to be involving inhibition of nuclear translocation of NF-<kappa>B and its binding to NF-<kappa>B consensus sequence. Octacosanol is a new antiangiogenic and antitumor agent that may lead to more selective and less toxic antineoplastic therapy.

The dried plant powder of T. cordifolia was extracted sequentially from non-polar to polar solvents namely hexane–benzene–chloroform–ethyl acetate and methanol. The solvents were evaporated by rotary evaporator and all the fractions were tested for antiangiogenic activity by peritoneal angiogenesis and Chicken chorioallantois membrane assay. Column was packed with hexane using silica gel 100–200 mesh size as a matrix and the hexane fraction

was loaded as dried slurry of silica gel. The ratio of material loaded and silica gel was 1:20. Elution was performed by hexane/chloroform/acetone (7:2:1) as mobile phase and all the eluted fractions were subjected to thin layer chromatography analysis. All the fractions eluted from the column were tested for bioactivity. One of them, fraction F 4, which showed antiangiogenic activity was further purified using HPLC Vydac C18 column in a Shimadzu LC-10AVP system with dual wavelength detector. The column was equilibrated with HPLC grade water and the loaded compound was eluted using linear gradient of 100%methanol at a flow rate of 1ml/min. The active compound was subjected to structure elucidation by ^{1}H, ^{13}C and $^{1}H–^{13}C$ COSY NMR spectroscopy and mass spectroscopy. The NMR experiments were done in $CDCl_3$ solution. Octacosanol (10mg) was dissolved in 100 µl of chloroform and 900 µl of DMSO and diluted 10 times with sterile distilled water to make final concentration of 1 µg/µl and used for subsequent experiments.

15.Extraction of Sugarcane Wax from Press Mud Solvent Extraction

Sugar cane press mud waste was extracted with different solvent such as Toluene and Benzene under a reflux system for 4 – 6 hr. at a stretch. The extract was filtered under mild vacuum and solvent recovered by distillation. After recovering the solvent, the solid mass containing wax mixtures and resins thus obtained was dissolved in hot isopropyl alcohol and filtered. The resin portion was separated and the total wax portion obtained which yellow or light cream was in color. The Physico-chemical properties of wax were analysed by Saponification, Iodine and Acid value, which were determined using standard methods of BIS (Lamberton et al., 1960).

The physico-chemical properties of press mud before and after extraction of wax were analysed. It includes pH, Moisture content, Total Nitrogen, Phosphorus, Potassium, Organic matter, organic carbon, Calcium, Magnesium and C: N ratio. All the procedures were followed described in APHA (*American Public Health Association)*.

16.Supercritical Extraction of Policosanol from Sugar Cane Wax

Sugar Cane Wax is obtained by heptane extraction from the sugar cane filter mud, a residue resulting from the sugar cane production containing 75% water, a large variety of fats, waxy esters, free alcohols, sterols and a resinous fraction mainly composed of calcium salts of heavy polyesters (Garcia et al., 1988). The industrial process for the separation of these alcohols consists of different successive and multiple steps, permitting a first fractionation of resin compounds, and the separation in a second step, of fats and a refined wax. Further solvent processing of the refined wax is focused on the isolation of a natural mixture of high molecular weight aliphatic alcohols of the series $C_{24}–C_{34}$, which once purified, have medical application like a medicant called Policosanol. This long current process has several problems related to both, complexity and the use of organic solvents (toxic, expensive, generation of residues, low selectivity) requiring solvent recovery, as well as being energy-intensive operation.

The use of CO_2 under supercritical conditions has been expanded to the isolation of bioactive compounds from natural materials like lanolin, jojoba esters and popolis (Lagunas et al., 1992). Due to CO2's properties, selectivity of triglycerides and waxes has also been achieved for substances containing a high level of lipid material (Stahl et al., 1988). This procedure has been used for the separation of natural waxes (Stahl et al., 1985) and aliphatic primary alcohols from the sugar cane wax (Fragernas, 1986) and rice bran (Garcia et al., 1994). All these results indicate that supercritical CO_2 technology is a promising alternative to the actual organic solvent extraction of the long chain alcohols from sugar cane wax.

Supercritical fluid extraction (SFE) as a technically viable alternative process for the extraction of high molecular weight *n*-alcohols from the sugar cane wax (Furukawa et al., 1987). Analysed the effect of pressure (300–350 bar),

temperature (50–100°C) and the amount of KOH used in the previous saponification (1–20%, w/w) on extraction yield of long chain material alcohols. A Response Surface Methodology (RSM) based on the statistical analysis (Lucas et al., 1997) of the experimental data was used to obtain mathematical expressions relating the operational variables and extraction yield.

17.Synthesis of 1-octacosanol

Policosanol was described by Cuban researchers as a mixture of eight higher aliphatic primary alcohols obtained from the wax of sugarcane (Saccharum officinarum L.). It contained: 1-tetracosanol (C_{24}), 1-hexacosanol (C_{26}), 1-heptacosanol (C_{27}), 1-octacosanol (C_{28}) – the most Abundant (60–70%), 1-nonacosanol (C_{29}), 1-triacontanol (C_{30}), 1-dotriacontanol (C_{32}) and 1-tetratriacontanol (C_{34}). The relative abundance of these alcohols and their total content defines the identity of policosanol. These mixtures are analysed by gas chromatography coupled with mass spectrometry (GC-MS) (Sierra et al., 2002)

The main step was a Wittig reaction between an octadecyl triphenyl phosphonium ylide and a methyl 10-oxodecanoate. In agreement with a flow-chart study of our synthetic process Fig. 2 (Cravotto & Cintas, 2007; Palmisano et al., 2007) (Cravotto et al., 2008).

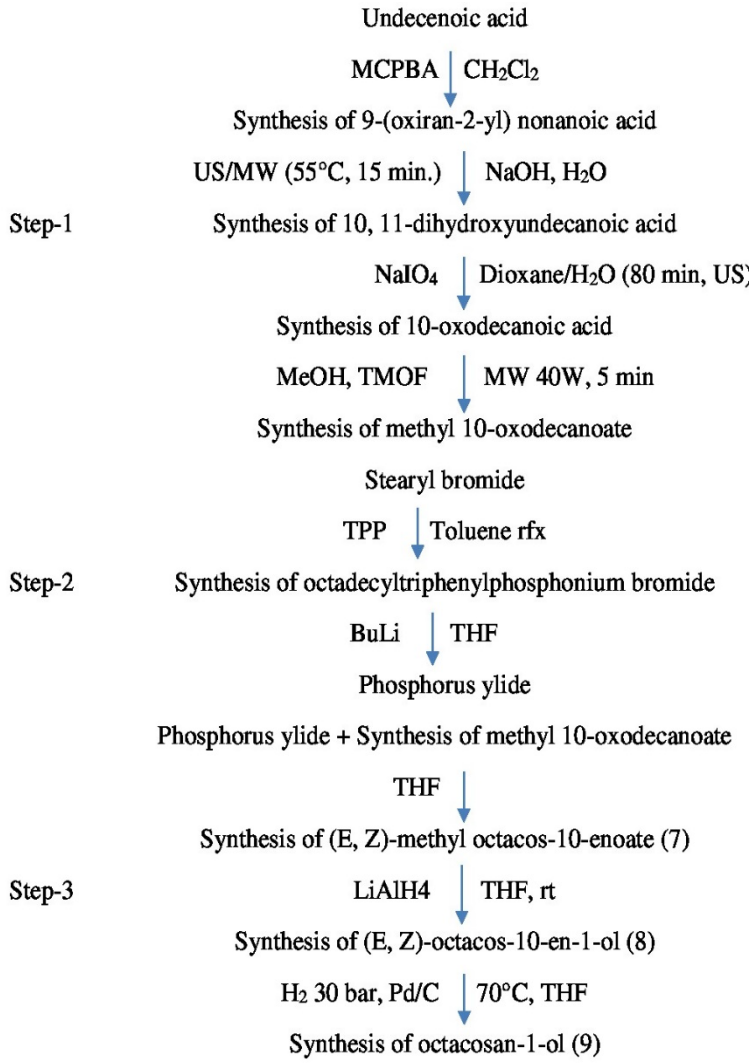

Figure 2. Synthesis of 1-octacosanol.

18.Extraction Methods for Policosanol from Rice Bran Wax

The effectiveness of different extraction methods for extracting Policosanol, especially Octacosanol, five extraction methods (A-E) were applied to the raw rice bran wax from manufacturer.

Method A. Saponification in alcohol was performed on the basis of the previously described procedure (Jiao & Wang, 2002) with some minor modifications. Briefly, 5 g of rice bran wax was mixed with 25 mL of 95% ethanol and 0.5 g of NaOH in a 100 mL four-hole flask and subsequently hydrolysed by refluxing in a water bath for 8 h with continuous stirring. Ten millilitres of alcoholic $CaCl_2$ solution (12 g of $CaCl_2$ plus 200 mL of 95% ethanol) was added, and the mixture was filtered while it was hot. After two washings with 95% ethanol, the mud cake was discarded. The collected filtrates were combined, cooled, and filtered again. The mud cake obtained in this step was dissolved with 3 times the volume of acetone preheated at about 50 °C. The solution in acetone was filtered after cooling. The resultant white PC product was naturally dried at ambient temperature.

Method B. Saponification in water (non-neutralized) was performed on the basis of a previously described procedure (Li & Qian, 2003) with some minor modifications. Briefly, 5 g of rice bran wax and 3 times the volume of water were placed in a 100 mL four-hole flask. After the wax was melted at 85 °C in a water bath, 0.5 g of NaOH was added. The mixture was boiled for 12 h followed by heat preservation for 36h. One millilitre of saturated $CaCl_2$ in water was added, and the reaction was continued for an additional 3 h at 80 °C. After filtration, the mud cake was washed to neutrality with hot water (80 °C) and dried at 65 °C. Subsequently, the solid was loaded to a Soxhlet apparatus and was extracted with 6-8 times the volume of acetone (Liu et al., 2001) as solvent for 16h. Once the extract solution had cooled, the Policosanol product was crystallized. Then it was filtered and dried at 65 °C.

Method C. Saponification in water (neutralized) was performed on the basis of the previously described procedure (Xu, 2002a) with some minor modifications. Briefly, 5 g of rice bran wax, 35 g of water, and 0.75 g of NaOH were placed into a 100 mL four-hole flask followed by continuous stirring for 20 h at 98 °C in a water bath. Ten grams of 10% HCl solution was subsequently added to neutralize the resultant. After 10 g of 8% $CaCl_2$ solution had been added, the reaction was kept for an additional 3 h. The resultant was cooled and filtered. After drying, the solid was refluxed with 10 times the volume of acetone for 12 h. The extract solution was cooled and filtered again. Policosanol product was obtained after drying.

Method D. Dry saponification was performed on the basis of the previously described procedure (Xu, 2002b) with some minor modifications. Briefly, 10 g of rice bran wax and 3 g of 50% $Ca(OH)_2$ soliquoid in water were added to a 100 mL four-hole flask and heated at around 100 °C in a water bath with continuous stirring for 5 h. The brown resultant was solidified once cooled. After the solid, which is the mixture of APAs and Ca-SFAs was weighed and ground, 5 g of the powder and 75 g of 95% ethanol were transferred into another flask and refluxed for 2h under stirring. The mixture was filtered while it was hot. After the filtrate had cooled, the Policosanol product was crystallized from the filtrate and was filtered and dried at 65 °C.

Method E. Trans esterification was performed on the basis of the previously described procedure (Chen et al., 2003) with some minor modifications. Briefly, 5 g of rice bran wax and 50 mL of 0.1% KOH solution in *n*-butanol were placed into a 100 mL four-hole flask. After 8 h of refluxing with continuous stirring, the reactant was cooled and substantially filtered. The mud cake was washed with hot water until it was neutral. After drying, the mud cake was loaded into a Soxhlet apparatus and extracted with acetone as the solvent for 12 h. The extract solution was cooled and filtered. The PC product was obtained after drying.

19.Conclusions

The major compound in Policosanol is 1-Octacosanol which is a long chain primary alcohol which has been obtained by various sources like animal, plant and synthetic route also. For isolation and purification various techniques used like supercritical fluid extraction, solvent evaporation extraction etc. and octacosanol quantification performed by gas chromatography.

20.References

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