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Research Article

A CONVENIENT AND INDUSTRIALLY VIABLE ROUTE TO SEPARATE LIPOPHILIC AND HYDROPHILIC FRACTIONS OF SEABUCKTHORN PULP AND ANALYSIS OF THEIR ACTIVITIES

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ABSTRACT

Traditionally, the extraction of oil from the Seabuckthorn pulp was done by first separating out the juice by mechanically pressing the berries followed by centrifugal separation of oil from the juice by density difference. But the pressed pulp cake still contained both the oil and the juice to some extent. Thus, a complete separation of the lipophilic (oil) and hydrophilic (juice) fractions of the pulp was not achieved. The process that we present in this work separates both the oil and juice fractions very discretely by directly processing the pulp as a whole in an alcoholic medium to separate the lipophilic and the vitamin-rich hydrophilic fractions embedded in the pulp. Moreover, we achieve this separation without employing expensive machines like mechanical presses and high-speed centrifuges. Further, the extraction of the oil from lipophilic part has been done by both the solvent and supercritical extraction methods followed by a comparative study using HPLC analysis of the bio-actives obtained from both the methods. We found that the carotenoids extracted better with the solvent extraction, while sterols extracted better with the supercritical extraction. The overall yield of the oil and the bio-actives was comparable to that of the earlier methods. The aqueous alcohol soluble fraction was evaluated for vitamin C, total acids and reducing sugars. We conclude that our process is convenient, cost-effective and good in terms of yield and recovery of the bio-actives, which makes it industrially applicable. This was endorsed by a successful pilot study trial of a 10 kg batch conducted at SSP India Pvt Ltd. Faridabad, India.

Keywords: Seabuckthorn; *Hippophae rhamnoides*; Supercritical Fluid (SCF) extraction; carotenoids; sterols; tocopherols

INTRODUCTION

SBT (*Hippophae rhamnoides L.*) (Elaeagnaceae) also known as seabuckthorn, is a thorny, deciduous, temperate bush plant widely distributed throughout the temperate zones of European and Asian countries and all over the subtropical zones, being found specially at high altitude of 2,500-4,300 m[1].Seabuckthorn is a berry producing shrub, of high economical value. Seabuckthorn (SBT) have been used for more than 1000 years in Tibetian and Chinese system of medicine[2]. Seabuckthorn leaves, berries, and bark have a high content of nutritive and active substances which promote the species for its use in both food and medicinal industries[3].Seabuckthorn is rich in antioxidant phytonutrients. Seabuckthorn contains a series of bioactive compounds including carotenoids, tocopherols, sterols, flavonoids, phenolics, lipids and ascorbic acid[4].

Seabuckthorn berries have tiny green/yellow flowers and orange pearl-shaped berries, which are not very sweet but have a mild unique aroma and a high content of vitamin C[5].The tremendous combination of the highest content of vitamin C, tocopherols and tocotrienols, phytosterols, and carotenoids makes the seabuckthorn berries an optimal raw material for functional foods having special anti-oxidative properties with anti-aging effect, due to stabilisation of cellular membrane[6].

One of the most requested therapeutic products on the market is seabuckthorn oil extracted from berries (pulp and seeds). Seabuckthorn oils are rich in fatty acids and vitamins. The various *in vivo* study of seabuckthorn oil reported to have anti-tumour[7, 8],antioxidant [9, 10], anti-microbial[11,12], anti-ulcer properties[8]and hepato-protective[8,13] and for treating cardiovascular diseases[8].The oil has several other pharmacological actions such as diminishing inflammations, relieving pain, reducing the toxic effects of traditional drugs, skin tissue regeneration after mechanical, chemical and burn injuries, used in skin grafting treatment of corneal wounds;anti-mutagen effects[12]. Besides its medicinal use, seabuckthorn berries can be processed into juices as a nutritional supplement.

Oil exists in both the seeds and pulp of the seabuckthorn berries. In the past, people have attempted oil extraction from both the seeds and the pulp[14- 16]. Our work here focuses on the pulp oil, which has been reported to contain more carotenoids than the seed oil and has high pharmaceutical value[17,18]. In earlier works, the oil extraction process started with the mechanical pressing of the berries to separate the juice, leaving the pulp cake behind[15]. The juice thus obtained was a turbid product still containing the oil portion[19] and required further separation. High speed centrifuges were used to further remove the oil by density difference[20], but still the complete separation was not achieved. Extraction of the oil from the pulp cake left after mechanical pressing was done by first drying the pulp cake to obtain flakes[16]. From the flakes, oil was extracted using four processes viz. solvent extraction using petroleum ether, supercritical fluid (SCF) extraction using CO_2, screw-processing, and aqueous extraction[16]. The yield of the oil and bio-actives has been reported to be best with the solvent extraction using petroleum ether.

In this work, we have adopted a different route for the extraction of oil from the seabuckthorn pulp. We do not perform mechanical pressing of the berries, instead, we directly process the pulp as a whole in an alcoholic medium to discretely

separate the lipophilic and the vitamin-rich hydrophilic fractions embedded in the pulp. From the alcohol-insoluble lipophilic fraction, we perform both the solvent extraction and SCF extraction with CO_2 to extract the oil at controlled temperature and pressure conditions so as to avoid the loss of bio-actives. From the hydrophilic fraction, we distilled off the alcohol to obtain a concentrate, rich in vitamins, organic-acids, and carbohydrates that can serve as a potent nutraceutical product, high in demand these days. Further, we did a critical analysis of the bio-actives obtained from both the solvent and supercritical fluid extraction of the oil followed by a comparative study of the yield. Also, the hydrophilic alcohol soluble portion was analysed for ascorbic acid content, reducing sugars, moisture-content and acidity content.
This process gives us a clean separation of the lipophilic and the hydrophilic fractions without employing costly machines like mechanical presses and high-speed centrifuges. Also, the yield of the oil and the bio-actives is comparable to the corresponding yield from the processes done by others in the past. Since the proposed process is simple, economical and has good yield, it is highly suitable to be used in industrial production. We tested its viability by doing an easy set- up of a pilot trial (10 kg batch) at SSP India Pvt Ltd. Faridabad. In addition, the use of ethanol as the medium for initial separation, coupled with SCF-CO_2 extraction makes the extracted oil fit to be used in the food industry.

MATERIALS AND METHODS

Berries of *Hippophae rhamnoides* were collected from Ladakh region of India and stored at -20^{0}C. To separate the seeds from the pulp, the frozen berries were passed through the pulping machine. The analytical standards were purchased viz. β-carotene from Sigma Aldrich Co. (St Louis, MO, USA), tocopherol standards from Merck & Co., Inc. (Darmstadt, Germany) and sterols from Matrix Fine Sciences Pvt Ltd., Aurangabad, India.

Seabuckthorn pulp extraction process

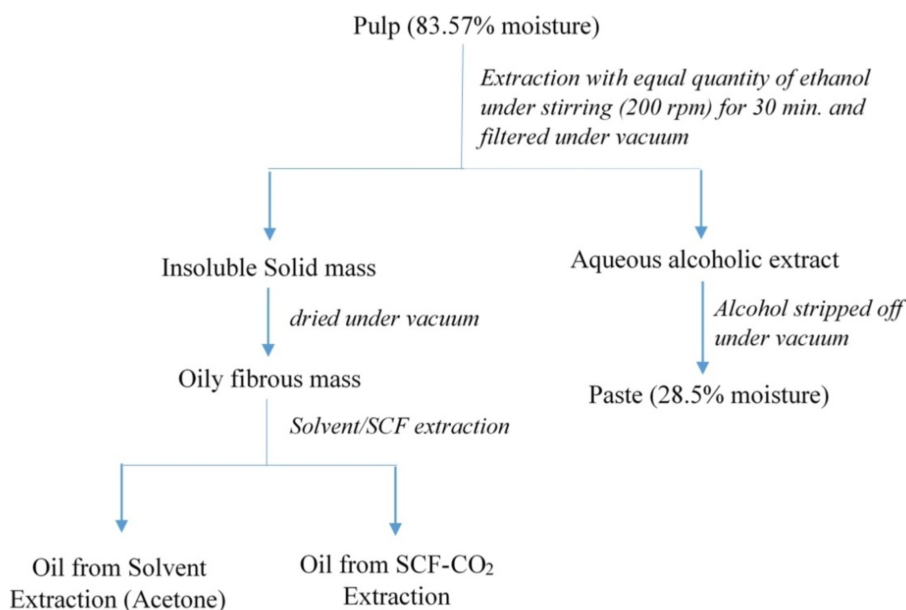

Figure 1: Seabuckthorn oil Extraction process flow

The extraction of seabuckthorn pulp oil has been done earlier, but we have done by adopting a different route. The process that we have adopted and described below is carried out at low temperature and pressure conditions to prevent the loss of antioxidant activity of the oil. 500 g pulp with moisture contents of 83.57% (Karl Fischer method) was transferred in a 5 l glass reactor fitted with a stirrer (200 rpm) and 0.5 l ethyl alcohol was added with stirring. The contents were stirred for 1 h and filtered on 300 mesh cloth. The pulp left on the cloth was again stirred with 250 ml ethyl alcohol for 30 min and filtered on 300 mesh cloth. The alcohol insoluble pulp obtained was reddish-yellow in colour and alcohol soluble fraction was pale yellow in colour.

Alcohol soluble Fraction*:* The alcohol was distilled off leaving behind 45g concentrate composed mainly of carbohydrates, organic acids and vitamins like B and C.

Alcohol insoluble Fraction: The alcohol insoluble portion was dried under vacuum at temperature 50^{0}C. This portion is then taken for solvent and supercritical extraction. Before extraction, the dried raw material (pulp) was ground to particle size of 2.4mm. The particle size distribution was determined by sieving (80% passed through a 10 mesh sieve)

By Solvent Extraction*:* The dried pulp 30 g was treated with 100 ml acetone and 100 ml n-hexane in a high shear mixer at room temperature for 2 h. It was then filtered under vacuum. The residue on filter paper was washed with hexane. The filtrate and washings were collected, and the solvent was distilled off under vacuum. This gave a dark yellowish red oil (yield = 18 g). The fibre after drying was obtained with a yield of 11.85 g. This process has been evolved by conducting series of experiments to extract oil. The fibre was obtained as a by-product.

By Supercritical CO_2 Extraction (SCF)*:* The ground plant material was extracted in a high pressure pilot plant with a 0.5 l extraction vessel, which was designed and delivered by Waters (Milford, USA). The extraction vessel was filled with about

100g of dried pulp. The desired extraction conditions with temperature 313K and pressure of 300 bars were set and the CO_2 feed was started. The CO_2 flow rate was measured with a mass flow meter. The specific flow rate was about 20mlCO_2 min^{-1}. Extract samples were collected and weighed every hour. Total extraction times were 6h.
To check if the above extraction was complete, we further processed the spent solid pulp obtained after the supercritical CO_2 extraction. The spent solid pulp was extracted with acetone at 45^0C (for 42 g of spent material, 200 ml acetone was used) for 2 h. The same process was repeated once more for complete extraction. The acetone extract was then pooled and evaporated under vacuum in rota-evaporator (Hydolf, Germany) to thick oily paste (14.6 g). The solid mass left was dried and weighted 22.3 g.
After the extraction of the oil, nanosizing and the determination of the colour units was done to incorporate the oil in cosmetic[21] and nutraceutical formulations. Nanosizing was done to have an enhanced spread and color in the final cosmetic formulation.

Nano-sizing of Seabuckthorn pulp oil
Seabuckthorn oil was solubilized in polyethylene glycol 400, Tween 80 and poloxamer which belongs to GRAS category. A ratio of 1:3 mixture of PEG 400 and water was prepared and sonicated using handheld ultrasonicator for 15 min at an amplitude of 1.0. Thereafter, the sample was sent for size analysis using Malvern zeta sizer. The average particle size was found to be 142.4 nm.

Determination of colour units by lovibond tintometer
The obtained oil was analysed for colour units

Red unit (Lovibond Tintometer, Model F, Visual)	5 (1.0% solution in sesame oil)
Yellow unit (Lovibond Tintometer, Model F, Visual)	22 (1.0% solution in sesame oil)

Determination of carotenoids, tocopherols and sterols in SBT pulp oil (solvent and SCF extracted) by high pressure liquid chromatography (HPLC)
The determination of tocopherols was carried out in accordance with ISO 9936:1997.23 standard. HPLC analysis was performed using the Agilent infinity 1220-series. HPLC system consisted of a pump, sampler, and an ultraviolet-visible (UV-vis) detector. The HPLC pumps, sample, column temperature, and UV Detector were monitored and controlled by using Agilent Instrument Utilities Software (QEZChrome). Samples for tocopherol determination were prepared by dissolving 102.2 mg of solvent extract in 10 ml of acetonitrile and 150 mg of SCF extract in 10 ml of acetonitrile. 10.5 mg of standard tocopherol is dissolved in 10 ml of acetonitrile. Samples for sterol determination were prepared by dissolving 501.6 mg in 10 ml methyl alcohol for both solvent and SCF extract and 100.5mg of standard sterol was dissolved in 10 ml methyl alcohol.

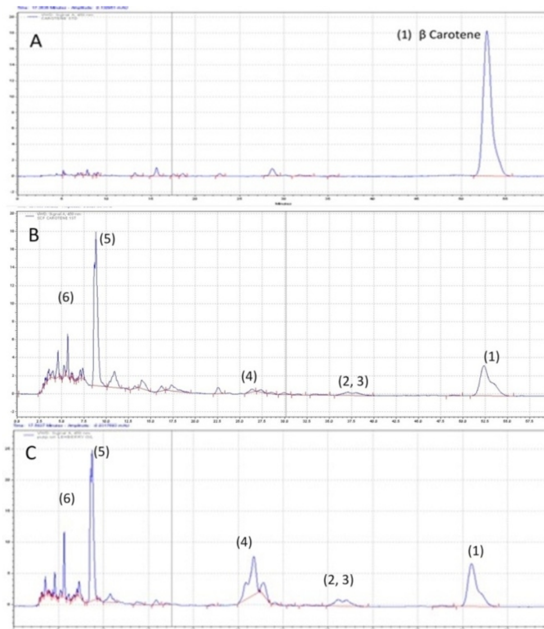

Figure 2: Chromatograms of the carotenoids: Standard (A), SCF (B) and Solvent (C) extractions. Peaks: 1: β-carotene, 2,3: ϒ and δ carotene, 4: Lycopene and its isomers, 5&6: unidentified

Samples for carotenoid determination were prepared by dissolving 212.5mg in 10ml mobile phase in solvent extract and for SCF, 216 mg in 10ml mobile phase. 1 mg of the β-carotene standard was dissolved in 10 ml mobile phase. Precisely 20 µl of the solution was injected onto an Eclipse plus C-18 column (4.6x250 mm, 5 µm particle size). Identification was made by comparison with external standards.

Table 1: HPLC equipment and conditions for carotenoids, tocopherols and sterols content analysis

Instrument, Column	Agilent infinity 1220, Eclipse plus C-18 (4.6x250mm)		
Condition(s)	**Carotenoids**	**Tocopherols**	**Sterols**
Mobile phase	Acetonitrile:Methylalcohol :Ethylalc 73:20:7(v/v) isocratic	MeOH: Acetonitrile 20:80(v/v)	Methyl alcohol
Run-time	60 min.	35 min.	25min.
Flow rate	1 ml/min.	1 ml/min.	1 ml/min.
Wavelength	450nm	280nm	210nm
Injection volume	20µl	20µl	20µl
Column Temperature	30^0C	30^0C	30^0C

.

Determination of Ascorbic acid in aqueous alcohol soluble fraction of seabuckthorn pulp by high pressure liquid chromatography (HPLC)
Samples of Ascorbic acid determination were prepared by dissolving 500 mg extract in 25ml mobile phase by sonication for 10min and then filtered through 2.5µ filter. 50 mg of ascorbic acid standard (Sigma Aldrich) was dissolved in 25ml mobile phase by sonication for 5 min and filtered through a 2.5µ filter.

Table 2: HPLC equipment and conditions for ascorbic acid content analysis

Instrument, Column	RPC_{18} (250*4.6mm, 5um)
Condition(s)	**Ascorbic acid**
Mobile phase	Methanol:Water 5:95(v/v)
Run-time	3.558 min
Flow rate	1 ml/min.
Wavelength	254 nm
Injection volume	20µl
Column Temperature	30^0C

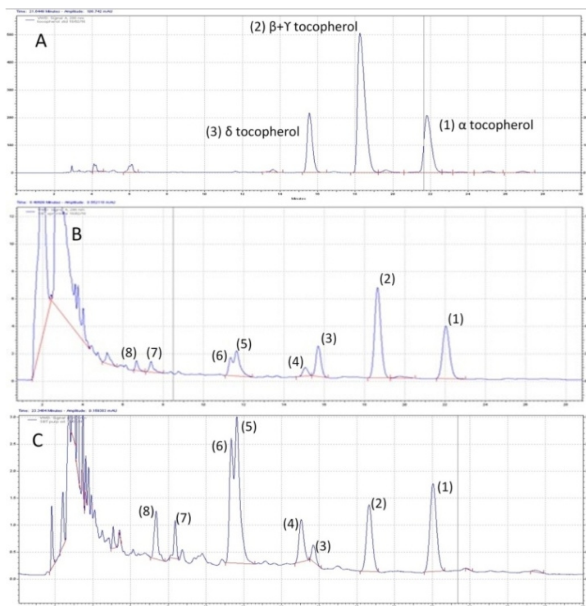

Figure 3: Chromatograms of the tocopherols: Standard (A), SCF (B) and Solvent (C) extractions. Peaks: 1: α tocopherol, 2: β+ϒ tocopherol, 3: δ tocopherol, 4: unidentified, 5,6,7,8: tocotrienols

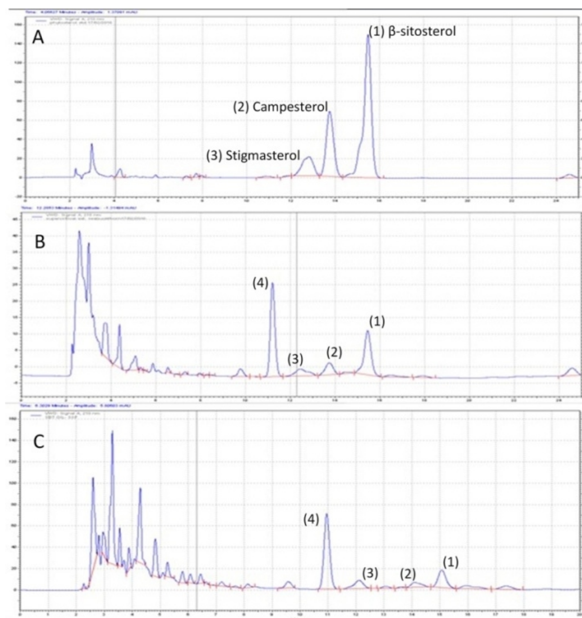

Figure 4: Chromatograms of the phytosterols: Standard (A), SCF (B) and Solvent (C) extractions. Peaks: 1:β-sitosterol, 2: Campesterol 3: Stigmasterol, 4: unidentified

RESULTS AND DISCUSSION

All the three fractions i.e. the oil, the aqueous juice concentrate and the dry fibre obtained from the process described in this paper were worked out for yield and the characterization of the actives. The hydrophilic concentrate separated from the pulp using ethyl alcohol was found to be 9%with respect to the raw material (i.e. the wet pulp). The yield of the oil obtained from lipophilic part was found to be 3.5% and 2.8% from solvent and SCF extraction respectively. The yield of the oil was slightly lower in SCF extraction, which led us to further process the spent solid pulp left from SCF extraction as described in the previous section. The obtained oil was analysed and characterization of the bio-actives was done with their HPLC profile.

Total tocopherols content in the pulp oil extracted by solvent extraction (hexane and acetone (1:1v/v)) was found to be 0.28% (283 mg/100 g oil) and with SCF-CO_2 extraction, it was 0.17% (178 mg/100 g of oil) for 6h extraction. In the earlier works[16], the total tocopherols concentration in the pulp oil was reported as 143.7 mg/100 g for the oil extracted in petroleum-ether as the solvent, while the concentration was 113.0 mg/100 g in the case of SCF-CO_2 extraction.The higher concentration of tocopherols in our case can be attributed to the different process used by us and also to the fact that we did the oil extraction from the wet pulp as against [16] who did it using the dried pulp flakes.

For total carotenoids, the concentration with our process in case of solvent extraction turned out to be 0.63% (637.5mg/100g oil), while with SCF-CO_2 extraction, it was 0.24% (245.6 mg/100 g oil, 6 h extraction).In the earlier works[16], the total carotenoid concentration in the pulp oil was reported as 527.8mg/100 g when extracted using petroleum-ether and 148.4 mg/100 g with SCF-CO_2 extraction. Again, the same reasons apply as for tocopherols, for explaining the higher concentration of carotenoids obtained in our case.

Finally, for sterols, the results looked a bit different for us in terms of the yield in case of solvent and SCF extraction. The average yield of sterols reached 628 mg/100g when SCF-CO_2 extraction was used. On the other hand, extraction with solvent afforded a total sterols content of 562 mg/100g of raw material. This difference can be easily noted from the HPLC chromatograms for sterols in Fig. 4. In the earlier works[16], the total sterol concentration in pulp oil was reported as 582 mg/100 g oil extracted by petroleum ether and by SCF- CO_2 extracted oil was 540 mg/100 g oil.

Comparing the solvent and SCF extraction processes, we can see that the sterols extracted better with SCF extraction, while carotenoids extracted well in the solvent. Among carotenoids, the lycopenes and its isomers were poorly extracted by SCF, while their recovery was appreciable in the solvent. The HPLC chromatograms in Figure 2 gives a full account of their distribution and recovery. In view of the above results, the solid pulp left after SCF extraction was extracted with the organic solvent (acetone) to recover extract that didn't get isolated with SCF. The analysis of the thus recovered extract showed both lycopene and carotene isomers were present in substantial amount while sterols were poorly present. Tocopherols and tocotrienols were almost equally extracted by both solvent and SCF extraction methods as can be seen in the HPLC chromatograms in Figure 3. Table 3 summarizes the results we obtained by both the solvent and SCF extraction methods.

Table 3: Analysis of SE and SCF-CO_2 extracted SBT pulp oil from lipophilic fraction

	Solvent extraction	Supercritical extraction
Refractive Index	1.47	1.46
Acid value	7.6	8.6
Specific gravity	0.82	0.82
Sterol (β-sitosterol) *(by HPLC method)*	0.56%	0.62%
Total tocopherols *(by HPLC method)*	0.28%	0.17%
Totalcarotenoids*(by HPLC method)*	0.52%	0.2%

Analysis of Aqueous alcoholic extract

The content of vitamin C was found out to be 8.5mg/g by HPLC from the aqueous alcoholic extract. In an earlier work[22], the content of vitamin C from pulp juice was reported as 0.02-0.2g/l. The table below summarizes our results of analysis of the aqueous alcoholic extract.

Table 4: Analysis of the aqueous alcoholic extract

Total acidity (calculated as citric acid)	21.25mg/gm of extract
Total reducing sugar equivalent to glucose	580mg/gm of extract
Moisture content in concentrate	34.36%
Ascorbic acid contents by HPLC	8.5mg/gm

Analysis of the dry fibre left after Solvent and SCF extractions

The crude and dry fibrous mass left after the solvent and SCF extraction was analysed for moisture, protein contents etc. Details are shown in Table 5.

Table 5: Analysis of the dry fibre left after oil extraction

Water soluble fibre	21.8%
Water insoluble fibre	64.35%
Protein contents (by Kjeldahl's method)	23.38% (Solvent extraction) 24.82% (SCF extraction)
Moisture contents	9.4%

CONCLUSION

Seabuckthorn berries are a unique source of dietary and medicinal supplements along with high valued oils (pulp and seeds).In this work, we have described an industrially viable process for seabuckthorn pulp extraction where we have taken a different approach as compared to the earlier work done in this regard. With our process, a clear separation of the three fractions of the seeds-free seabuckthorn pulp i.e. the oil, juice concentrate and the dry fibre is achieved. We perform the separation without making use of machines like mechanical presses and high-speed centrifuges which were used in the earlier approaches. This lowers the cost of setting up an extraction plant, making the process convenient and industrially viable. Further, we compared the yield and recovery of the bio-actives in the obtained fractions with those in the earlier approaches and found them to be comparable. Also, a comparison of solvent (hexane and acetone) and SCF-CO_2 extraction processes has been done with respect to the recovery of the bio-actives. Here, we concluded that the extraction done by SCF under described conditions is enough to extract all active components in the oil. Either pressure or temperature conditions are to be optimized or co-solvent will have to be utilized for a complete recovery of the actives like lycopene etc. The extracted oil was nano-sized for an enhanced spread and hue in the cosmetic formulations. The use of ethanol as the separation medium kept the suitability of the extracted fractions intact to be utilized in formulating nutraceuticals that we plan to do in future.

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