

Extraction of Essential Oil from Radhuni (*Carum roxburghianum*) via Novel Techniques with Process Intensification and Characterization

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Abstract: The therapeutic potential of Radhuni (*Carum roxburghianum*), an aromatic fruit-bearing plant, has been extensively studied through traditional solvent extraction. Current research explores two innovative methods for extracting essential oils (EOs): Ultrasound-Assisted Hydrodistillation (UAHD) and Dry Ice Fog Extraction (DIFE), compared with conventional hydrodistillation (HD). UAHD produced almost double the essential oil yield (0.94%) compared to HD (0.52%). Optimized UAHD with Response Surface Methodology resulted in a higher yield (1.64%) using specific parameters. The optimized UAHD batch exhibited superior quality compared to HD, characterized by a transparent, non-viscous appearance with a spicy, citrusy, woody scent. GC-MS analysis revealed predominant compounds like Limonene (56.61%), Sabinene (25.1%), and Ligustilide (3.3%). DIFE, primarily an isolation technique, yielded 16.67% extract with a citrus-peel alcoholic aroma. Sensory evaluation favored UAHD, followed by HD and DIFE. The remaining spice waste was converted into oleoresin (16.31%) and combusted for a calorific value of 23-27 MJ/kg as fuel.

Keywords: essential oil; Radhuni; UAHD; DIFE; RSM; GC-MS.

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1. Introduction

From ancient civilizations in China, India, and the Near East to modern times, plants have served as a fundamental source of medicinal remedies, forming an integral part of human healthcare practices. Despite the remarkable strides made in synthetic modern medicine over the last century, aromatic plants have garnered significant attention for their therapeutic potential and role in enhancing the quality of life. Aromatic plants, particularly spices, have enriched the flavors and aromas of foods and played a vital role in traditional healing practices, addressing chronic and infectious ailments [1].

Among these indigenous spices is *Carum Roxburghianum* (Benth and Hook), known as Ajmod in Hindi, Ajmoda in Sanskrit, Radhuni in Bengali, Karafs in Urdu, and Pak Chi Rai in Thai. This spice has a rich historical presence in Ayurvedic and Unani medicines, with references found in ancient texts like the "Charak Samhita" [2]. Taxonomically, this plant belongs to the Plantae kingdom, Tracheophyta phylum, Magnoliopsida class, Apiales order,

Apiaceae (Umbelliferae) family, *Carum* (*Trachyspermum*) genus, and *C. roxburghianum* species [3,4].

Carum roxburghianum, or Radhuni, is a fragrant herbaceous annual or biennial plant reaching 30–100 cm in height. It boasts feathery, well-developed leaves with white flowers and small, hispid, greyish-brown conical fruits [5–7]. Native to tropical Asian countries including India, Bangladesh, Pakistan, China, Thailand, Indonesia, and some parts of Africa, this spice shares lineage with Ajwain and is colloquially known as 'Wild celery' [8,9].

Culturally, Radhuni is a favored food flavoring, notably in Bengali, Eastern, and North-Eastern Indian cuisine, and is part of the "Panch Phoron" mixture. Beyond flavoring, its essential oil has applications in aromatherapy and as a component in "grip water" formulations. Traditional medicine attributes a range of benefits to Radhuni, from treating gastrointestinal issues to its potential as an anthelmintic, antimicrobial, and antioxidant. Yet, its incorporation into modern medicine warrants thorough clinical research and trials [10]. Driven by the need to enhance essential oil production profitability and extraction efficiency, this research aims to overcome the limitations of conventional extraction methods. Traditional hydrodistillation and solvent-based extraction suffer from drawbacks such as low efficiency, prolonged processes, oil quality degradation, and environmental concerns due to toxic solvents and waste generation. To address these challenges and align with the modern drive towards environmentally friendly practices and energy conservation, novel extraction techniques are being explored. One promising approach involves ultrasonic pre-treatment using acoustic cavitation followed by hydrodistillation, which offers rapid extraction, energy efficiency, and environmental compatibility [11-15]. Additionally, the study delves into Dry Ice Fog extraction, an innovative method that eliminates the need for time-consuming separation and purification steps [16].

Focusing on the relatively underexplored Radhuni fruit, this spice holds therapeutic potential that has been documented in previous studies, yet it remains largely unexplored in the context of novel extraction techniques (Figure 1). The primary objective of this research is to create a robust database concerning the oil of this spice [17-19]. This will be achieved by optimizing various extraction methods and subsequent comprehensive characterization of the extracted oil.

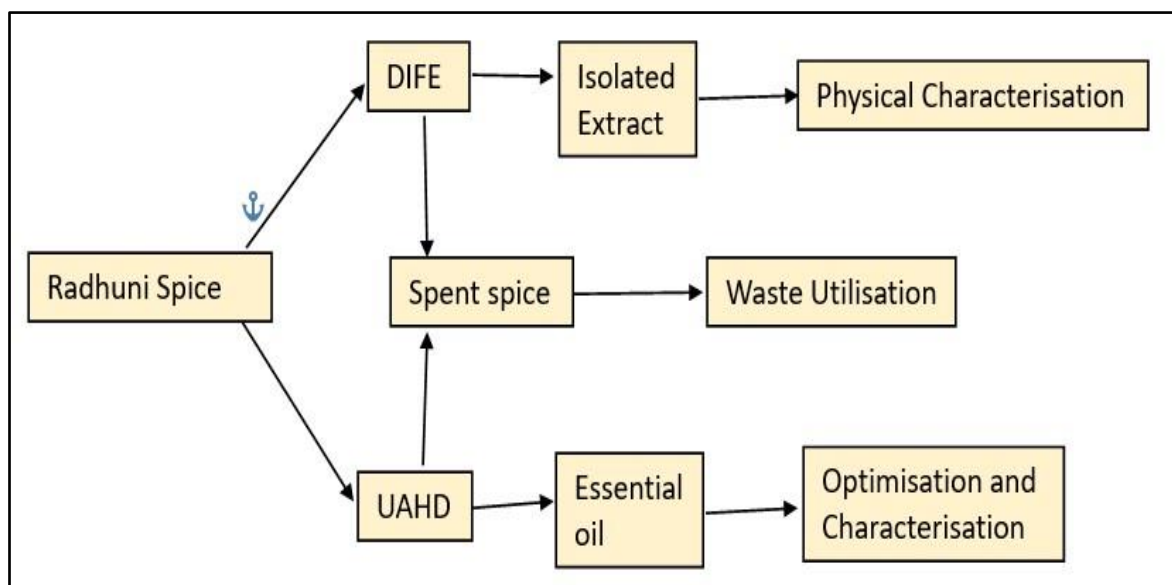


Figure 1. An outline of the research workflow.

2. Materials and Methods

2.1. Materials.

2.1.1. Spice Material.

The mature spice fruits were grown throughout the winter and spring seasons and harvested around May to June 2022 from a small village in the Nadia district of West Bengal, India. After harvesting, they were dried under mild sunlight for a day and passed through a gravity separator to remove contaminated stalks and stones. The spice was then accurately weighed and put into 50 g sealed air-tight LDPE-paper laminated pouches. All the processing was carried out under our surveillance and procured through a company called Amar Khamar.

2.1.2. Chemicals, Solvents, and Reagents.

The following solvents were required for various extraction and solubility tests, as well as other chemicals and reagents for analytical tests. Dry ice bricks were donated by the Naturals Icecream shop in Matunga Kings Circle. Acetone (99%, LR grade) and Methanol (99.8%, HPLC grade, 0.2micron filtered) were procured from SD Fine Chem Ltd. Petroleum ether (Extra pure, 40-60°C fraction), Chloroform (99.8%, AR grade) and Toluene (99.5%, AR grade) obtained from Loba Chemie Pvt Ltd. Commercial Hexane (85%, AR grade), Hydrochloric acid (HCl, 36%, LR grade), 2,2-diphenyl-1-picrylhydrazyl (DPPH, 95%), and Ascorbic Acid (AA, 99.7%, AR grade) were bought from Molychem. Ethanol (99.9%, LR grade) was imported from Changshu Hongsheng Fine Chemicals Ltd, China. Potassium Hydroxide pellets (KOH, 85%, AR grade) and anhydrous Sodium Carbonate (Na_2CO_3 , 98.5%, LR grade) were brought from generously donated by HiMedia Laboratories and Whatman filter paper no. 1 from GE Healthcare Life Sciences. Di-propylene Glycol (DPG, 95%) and Propylene Glycol (PG, 95%) were procured from Zeta Scientific. Lastly, anhydrous Sodium Sulphate (Na_2SO_4 , 98.5%, LR grade), Potassium Hydrogen Phthalate (KHP, 99.5% dry basis), Methyl orange solution, Phenolphthalein solution, and Isopropyl Alcohol (IPA, 90%, AR grade) from Thomas Baker Chemicals Pvt. Ltd.

2.1.3. Instruments, Equipment, and Glassware.

The various instruments used in this research are Gas Chromatography (GC, Rtx-5 column, Shimadzu GC-2014), Gas Chromatography Mass Spectrometry (GC-MS, DB5-ms column, Thermo Scientific Trace 1300), digital Abbe's Refractometer (Labman Scientific Instruments Pvt. Ltd.), Colorimeter (LabScanXE, HunterLAB, software EasyMatch QC version 4.95), Spectrophotometer (Jasco V-730, software Spectra Manager), Infrared Moisture Analyser (Ohaus MB23) and Bomb Calorimeter (Model CC01/M3, Toshniwal Tech Pvt. Ltd.). The other equipment includes, digital Weighing Balance (Anamed Instruments Pvt. Ltd.), Ultrasonic Bath (Model DF200, Dakshin Mumbai), Heating Mantle (300W, upto 400°C, SS, 1Litre, Labline Stock Centre), Mixer Grinder (750W, Philips), Chest Freezer (132W, BlueStar), Refrigerator (3-star, 215units/year, LG), Water Bath (1.1kW, Omkar Manufacturer), Hot Air Oven (2.5kW, Model HIPL023, Hexatech Instruments Pvt. Ltd.), Submersible Pump (VEGA, 18W, 1100L/hr capacity, Vardhman Electrical), Ice maker (Elanpro EIM41BW, 310W, 36kg/day capacity, Elan Professional Appliances Pvt. Ltd.), Precision water bath for DIFE (Model EV11.JIB.095, 800W, Medica Instruments Mfg. Co.), Water Chiller unit (Model

IC101, Escy Enterprises) and PID Temperature controller (230V±10% AC, Fourtech Systems). The various glassware like round bottom flasks, Clevenger, Soxhlet, and Dean-Stark apparatus with an attached condenser, thermo-pocket, funnel, entire customized DIFE system, conical flasks, air condensers, Petri dishes, test tubes, measuring cylinders, and volumetric flasks were procured from Borosil Ltd. The amber-colored 3ml, 10ml, and 30ml vials and bottles were brought from Bhagwandas Devshi Pvt. Ltd. of Kalbadevi, Mumbai.

2.2. Experimental Methods

2.2.1. Spice Preparation for Extractions.

For initial trial batches spice samples were weighed immediately after removing from sealed packs and extracted in UADH and DIFE. After that, some batches were taken by grinding the spice and extracting it. It was then optimized that 45g (for HD and UAHD) and 15g (for DIFE) on dry basis weight were previously weighed after moisture analysis and kept sealed in 250ml round bottom flasks with a stopper. The accurately weighed spice in a bottle was stored inside a Chest Freezer at -18°C overnight for 12 hours to freeze the whole spice matter. In the morning, it was ground using a Philips mixer grinder at 1 power setting for 15 seconds. The contents were then transferred from the grinder bowl to the extractors. To avoid spice loss in the bowl, a complete and clean transfer was ensured. In the case of HD and UAHD, a measured quantity of water was added to the bowl, spun, and transferred to the 1-liter round bottom flask for extraction.

2.2.2. UAHD and Conventional HD Techniques.

To the spice material whole or ground taken in a 3-neck round bottom flask, altered water (solvent) quantities were added in the required ratio to the spice (solute) amount. The flask was then stopped and placed in the Ultrasonic Bath. The fluid medium used in the bath was water, which was previously set and conditioned to reach the desired temperature before placing the round bottom flask, as observed in the picture in Fig. 3.2. given below. At a set frequency of 25kHz - the power from 160-220W, the ultrasonication temperature from 40-60°C and time for 30-90min was tested. When the ultrasonication pre-treatment was complete at a particular time, temperature, and power setting, the flask was removed from the bath, wiped to remove water on the outside surface, and put on a heating mantle for final hydrodistillation. On the main B24 neck, the Clevenger apparatus was attached to which, with a B19/B24 connector, a spiral condenser was connected. On a B19 neck of the flask, a thermo-pocket containing silicone oil (a good conductor of heat) was placed, and a temperature control probe connected to the PID temperature controller was placed. The controller was attached to the Heating mantle to cut off or power on the mantle according to the desired temperature setting on the controller. The other B19 neck was kept stopped to avoid and minimize volatile mass and energy losses. On top of the condenser, cotton was plugged in to check for volatile oil losses. The condenser was attached to the Submersible Pump in a bucket full of chilled water to recirculate the water. The condenser temperature was kept at approximately 15-18°C with ice chips from the Ice Maker to ensure complete condensation of volatiles. After the hydrodistillation was complete, the setup was allowed to cool down for 2 hours to find a clear separation of essential oil from water. Then, with approximately 0.02g cotton and 0.2g anhydrous Na₂SO₄, a hygroscopic bed was created on a small funnel through which the essential oil was passed and collected in 3ml amber-colored bottles with an inner seal. The

weight of the bottles before and after filling them with oil was measured to calculate the weight of oil, and the oil yield was considered in grams. It was then stored inside the refrigerator at 4°C. An exactly similar procedure was followed for conventional HD using both whole and ground spice without the ultrasonication pre-treatment and extraction time of 4.5 hours. The basic procedure for hydro distillation of spices was followed from ISO 6571:2008/Amd 1:2017 with few modifications.

2.2.3. DIFE Technique.

After a series of trials, the system design was customized based on the maximum surface area for better extraction and optimized headspace for retention of fog over the spice for adequate extraction. Describing the setup, a fog generator unit with 2 B40 necks (one on top and the other on side) was partially merged in the 4litre water bath, specially controlled for this technique. This vessel contained a solvent system for fog generation heated up to the required temperature set at the water bath. The side neck is initially kept closed with a stopper. Above the fog generator is the 15cm diameter extractor vessel with a B24 neck lid and 1 cm thick porous ceramic bed. The lid is usually sealed with Teflon tape, and the metal wire is tightened with a screw-nut. A bent neck spiral condenser is attached through the lid, connecting with a 250ml 2-neck round bottom flask receiver. With the other neck of the receiver, another straight-neck spiral condenser is attached above, and an air venter is attached to let out excess pressure and allow the forward flow of fog. The condensers are connected together and attached to a water chiller cum pumping unit to circulate chilled water. With the whole setup in place, weighed samples of ground spice were put into the extractor and sealed. Then, at the desired temperatures of a water bath and chilling unit, dry ice brick was pelletized with a hammer and loaded into the fog generator from the side neck. As the pellets come in contact with the solvent system, instantaneous fog is produced, which travels upward through the ground spice matrix, stays in the headspace for a few seconds, and then gets condensed in the receiver via the condensers, as shown by Fig. 3.6. The 2nd straight condenser is added as an extra measure to ensure negligible losses and maximum condensation. After extraction, the setup is dismantled, and the extract is collected and stored in 3ml amber-colored vials inside the refrigerator at 4°C.

To decide on the extracting solvent system, various polar and non-polar solvents like hexane, petroleum ether, chloroform, acetone, methanol, and ethanol were experimented with in different ratios with water. In a 250ml measuring cylinder, a ratio of water with other solvents was taken, and approximately small pellets of equal sizes were added to the cylinder to check the upward flowability and density of fog qualitatively [54].

After different trials and a literature survey, the extraction conditions were set as follows – 15-minute run time, bath and condenser temperature at 40°C and 5-6°C, respectively, a ground sample of 15g, ethanol to water ratio 1:4 and 200ml total solvent volume.

3. Results and Discussion

3.1. Initial trials on HD and UAHD treatment.

In the initial studies, a tussle to choose whole spice for extraction or grinding existed. Experiments were conducted to see if HD and UAHD treatment of whole and ground spice made any difference in extraction efficiency. By conducting trials in triplicates, it was found that UAHD treatment for ground spice, according to Table 1. below, yields a maximum of

0.94% (as ml/100g of spice), and HD treatment of whole spice yields a minimum of 0.39%. When ground spice was subjected to HD treatment, a 0.55% yield was obtained.

Table 1. Yield comparison of whole and ground fruit in HD and UAHD.

	HD	UAHD
Whole	0.154±0.02g (0.39%)	0.197±0.02g (0.52%)
Ground	0.212±0.03g (0.55%)	0.358±0.02g (0.94%)

The HD experiments were conducted with 45 g of spice (dry basis), 600 ml of water, 98°C, and an extraction time of 4.5 hrs. Whereas the UAHD had the same quantities of spice and water, and when pre-treatment was done at 25 kHz, 230 W, 50°C and 30 min, at 98°C extraction was completed by 2 hrs (observed with no change in oil amount in Clevenger). Furthermore, it was observed that while grinding the spice kept at room temperature, volatile losses were happening, and so to reduce that, the idea of freezing the accurately weighed spice overnight was implemented. No difference in yield or quality of oil was observed when extracted with distilled water and normal tap water. So, to save energy, further extraction experiments are conducted in tap water. A comparison of two main compounds as per gas chromatography results was made further to corroborate the usage of ground spice in research. According to the aforementioned UAHD parameters, the extracted oil was analyzed via gas chromatography. Compound A (Sabinene) and Compound B (Limonene) had higher relative content when extracted with ground fruit, as shown in Table 2. It means that these compounds are difficult to extract from the oil sacks of fruit and require the aid of mechanical grinding to increase the extraction surface area.

Table 2. GC relative composition of UAHD of whole and ground fruit.

	Compound A (Sabinene)	Compound B (Limonene)
Whole	14.71%	53.46%
Ground	23.12%	54.07%

After accurately weighing 45 g (dry basis) of spice, refrigerating overnight, and grinding it, HD of Radhuni spice yielded 0.78% oil (mL/100 g spice) at 98°C and extraction time of 4.5 hrs. In conclusion, UAHD pre-treatment and extraction takes 2.5 hrs and extracts more good quality oil at lesser time-temperature conditions, making the process economical.

3.2. Response surface methodology of UAHD treatment.

Extraction of Radhuni essential oil is dependent on factors like ultrasonication frequency, power, time, temperature; extraction time, temperature; and water-to-spice ratio. Ultrasonication frequency was kept constant at 25 kHz as it gave better cavitation and thus yielded better. Under the above-mentioned conditions of UAHD treatment, Voltage and thus Power of ultrasonication were varied, and it was observed that max power at 220 V gave a max oil yield of 0.358 g, as seen in Figure 2.

The extraction temperature was fixed at 98°C after several trials. A temperature below that did not volatilize and condense into the collector section of the Clevenger apparatus and a higher temperature gave impurities in oil like the volatile saponins, making the oil visible turbid pale-yellow. With all these parameters being fixed, the RSM study was conducted with a Sonication Time (STime) range of 30-90 min, Sonication Temperature (STemp) range of 40°-60°C, Water to Spice ratio (W-S ratio) range of 7-15 and Extraction Time (ETime) range of 30-120 min by trials. The basis of using statistical methods like RSM is that optimizing each of the 4 variable parameters/ factors individually is time-consuming.

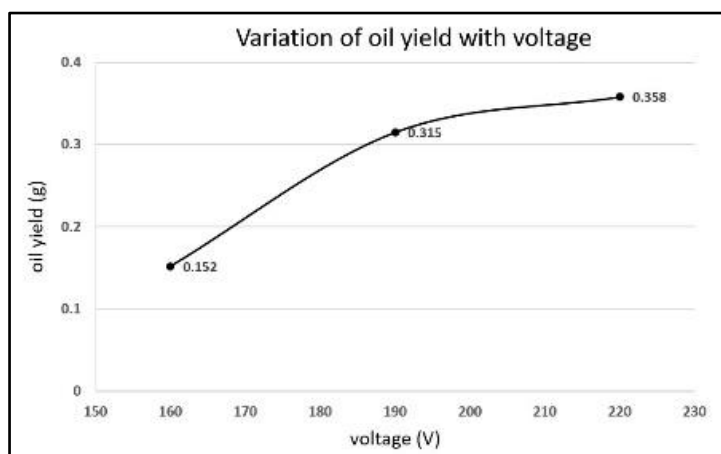


Figure 2. Variation of essential oil yield with voltage.

It is also used to understand the interaction between the parameters (independent variable) affecting the responses (dependent variable). The 4 responses, like oil yield and relative area of PeakA, PeakB, and PeakC, are to be optimized for variation in the input variable/ factor levels. The Box-Behnken Design (BBD) is used to optimize the effect of individual and interaction of input factors on the output responses. A design was generated using Design-Expert 13 software based on the given range of factors. The design had a total of 29 runs, with 5 runs at the center point of the design. The oil yield and relative areas of the peak via gas chromatography were measured according to the methods stated before. Table 3 below shows the design summary and the responses obtained in every 29 standard run.

Table 3. RSM design summary with responses.

Standard No.	STime (min)	STemp (°C)	W-S Ratio	ETime (min)	Oil Yield (g)	PeakA (%)	PeakB (%)	PeakC (%)
1	30	40	11	75	0.6104	25.602	56.071	3.642
2	90	40	11	75	0.4853	25.59	55.238	3.592
3	30	60	11	75	0.4479	26.382	56.923	2.108
4	90	60	11	75	0.4936	24.475	55.989	3.146
5	60	50	7	30	0.4496	26.172	56.991	2.671
6	60	50	15	30	0.3952	26.375	55.593	2.665
7	60	50	7	120	0.7063	24.261	53.708	5.776
8	60	50	15	120	0.5273	24.955	54.497	4.314
9	30	50	11	30	0.2837	27.598	59.139	0.767
10	90	50	11	30	0.3763	26.643	56.665	2.466
11	30	50	11	120	0.5631	25.972	55.928	4.013
12	90	50	11	120	0.4079	24.778	56.185	3.128
13	60	40	7	75	0.6731	25.238	55.298	5.064
14	60	60	7	75	0.6552	23.812	53.591	4.436
15	60	40	15	75	0.5889	25.021	53.024	4.561
16	60	60	15	75	0.5207	25.087	55.148	3.656
17	30	50	7	75	0.6126	25.536	55.446	4.218
18	90	50	7	75	0.5463	25.005	55.67	3.573
19	30	50	15	75	0.4904	26.576	55.846	2.777
20	90	50	15	75	0.4609	25.375	54.712	3.721
21	60	40	11	30	0.427	25.482	56.105	2.827
22	60	60	11	30	0.4583	26.806	56.946	1.896
23	60	40	11	120	0.6552	25.727	55.024	5.139

Standard No.	STime (min)	STemp (°C)	W-S Ratio	ETime (min)	Oil Yield (g)	PeakA (%)	PeakB (%)	PeakC (%)
24	60	60	11	120	0.5172	23.752	55.208	3.842
25	60	50	11	75	0.569	25.341	54.749	4.353
26	60	50	11	75	0.5724	25.313	54.63	4.524
27	60	50	11	75	0.5299	24.963	54.629	3.768
28	60	50	11	75	0.5844	25.31	55.292	4.121
29	60	50	11	75	0.5407	25.635	55.278	3.923

3.3.1. Statistical modeling with response ANOVA.

The experimental response data from the previous Table was used to make an individual statistical model correlating the factors to responses like oil yield and relative area of PeakA, PeakB, and PeakC. Various models such as linear, 2FI, quadratic, and cubic were tested with the help of Analysis of Variance (ANOVA), which suggested that the Quadratic model correlates the data best for the above output parameters compared to the other models. The quadratic model equations suggested for different responses are mentioned below in Equations 1.1., 1.2., 1.3., and 1.4.

$$\begin{aligned} \text{Oil yield} = & 0.804507 + 0.005267 * STime - 0.026654 * STemp - \\ & 0.047499 * Ratio + 0.016627 * ETime + 0.000142 * STime * STemp - 0.000045 * \\ & STime * ETime - 0.000094 * STemp * ETime - 0.000173 * Ratio * ETime - \\ & 0.000080 * STime^2 + 0.000223 * STemp^2 + 0.002124 * Ratio^2 - 0.000036 * \\ & ETime^2 \end{aligned} \quad (1)$$

$$\begin{aligned} \text{PeakA} = & 15.4105 - 0.0073 * STime + 0.4037 * STemp - 0.0678 * Ratio \\ & + 0.0441 * ETime - 0.0015 * STime * STemp + 0.0093 * STemp \\ & * Ratio - 0.0018 * STemp * ETime + 0.0006 * STime^2 - 0.0029 \\ & * STemp^2 - 0.0149 * Ratio^2 + 0.0002 * ETime^2 \end{aligned}$$

$$\begin{aligned} \text{PeakB} = & 74.8048 - 0.1734 * STime - 0.2380 * STemp - 0.4170 * Ratio \\ & - 0.1520 * ETime - 0.0028 * STime * Ratio + 0.0005 * STime \\ & * ETime + 0.0239 * STemp * Ratio + 0.0030 * Ratio * ETime \\ & + 0.0012 * STime^2 - 0.0398 * Ratio^2 + 0.0004 * ETime^2 \end{aligned}$$

$$\begin{aligned} \text{PeakC} = & 3.8326 + 0.0812 * STime - 0.1022 * STemp - 0.6473 * Ratio + 0.1204 \\ & * ETime + 0.0009 * STime * STemp + 0.0033 * STime * Ratio \\ & - 0.0004 * STime * ETime - 0.0020 * Ratio * ETime - 0.0010 \\ & * STime^2 + 0.0234 * Ratio^2 - 0.0003 * ETime^2 \end{aligned}$$

In the Design-Expert software, the Actual Equations predict responses based on input factors, but their coefficients vary with factor units, hindering relative impact analysis. Coded Equations, another equation set in normalized form, are independent of unit scales and help gauge factor effects. Coefficients' magnitudes and signs indicate factor importance and dependency. Analysis of Variance (ANOVA) explains response variance due to variables and interactions. ANOVA summary (Table 4) displays R² values—0.9773, 0.9678, 0.9680, 0.9780—for Oil yield, PeakA, PeakB, and PeakC, indicating a strong fit of quadratic models. High Adjusted R² signifies a robust correlation between experimental and predicted data, while elevated Predicted R² assures predictive accuracy. F-value, representing factor mean-variance to Residual variance, highlights factors impacting response variance. Higher F-values reflect a greater influence of input parameters. Firstly, the sum of squares for each factor and their

interaction is calculated individually. The following equations 1.5. and 1.6. then lead to an F-value.

$$\text{Mean square} = \frac{\text{Sum of squares}}{\text{Degree of freedom (df)}}$$

$$F \text{ value} = \frac{\text{Model or term mean square}}{\text{Residual mean square}}$$

The model F-value of 57.52, 46.48, 46.73, and 68.78 for Oil yield, PeakA, PeakB, and PeakC, respectively, implies that the model is significant and there is only a 0.01% chance this high F-value could occur due to noise. According to the p-values, values less than 0.05 indicate that the terms are significant, and values above 0.1 indicate that the terms are insignificant. In the model construction, insignificant terms have been removed to improve model efficiency. The significant terms are shown in Table 4. The insignificant ones like AC, BC in Oil yield; AC, AD, CD in PeakA; AB, BD in PeakB; and BC, BD in PeakC have also been removed from the models because their F-value was negligibly small compared to other terms. Here A = STime, B = STemp, C = W-S Ratio, D = ETime. Thus, the Models can be called Modified Quadratic Models.

Table 4. Summary of ANOVA with respect to the responses.

Factor	Oil Yield	Peak A	Peak B	Peak C
R ²	0.9773	0.9678	0.9680	0.9780
Adjusted R ²	0.9604	0.9470	0.9473	0.9638
Predicted R ²	0.9247	0.9043	0.9067	0.9458
Adequate precision	31.7928	30.4260	34.3908	38.0853
Model fit	Significant	Significant	Significant	Significant
Model F-value	57.52	46.48	46.73	68.78
Model p-value	<0.0001	<0.0001	<0.0001	<0.0001
Significant terms	A, B, C, D, AB, AD, BD, CD, A ² , B ² , C ² , D ²	A, B, C, D, AB, BC, BD, A ² , B ² , C ² , D ²	A, B, C, D, AC, AD, BC, CD, A ² , C ² , D ²	A, B, C, D, AB, AC, AD, CD, A ² , C ² , D ²
Lack of Fit	Not significant	Not significant	Not significant	Not significant
Lack of Fit F-value	0.6061	0.6208	0.5409	0.2587
Lack of Fit P-value	0.7743	0.7696	0.8203	0.9722

Comparing the F-values of terms individually, we see that D (Extraction Time) has been constantly of the highest significance in all response's maximum for PeakC. This is because a higher extraction time would lead to higher oil extraction and a higher relative area of PeakC, which is less volatile than the other 2 peaks/compounds. For the oil yield, the W-S ratio and square of STime are of 2nd most importance, showing that a quadratic relation with STime is more important than a linear relation. Similarly, the significance of each term on their corresponding responses can be seen in Table 5, implying that no single input variable can solely explain the variance in responses, and the interaction terms are equally important to be considered. The Lack of fit F-value implies that lack of fit is not significant to pure error and suggests that the model fits significantly.

Table 5. Terms and F-values of responses.

Terms	Oil Yield	PeakA	PeakB	PeakC
A	12.70	69.58	26.49	8.97
B	27.05	11.38	10.25	66.99
C	97.76	23.42	3.93	33.24
D	218.79	191.86	131.13	339.30

Terms	Oil Yield	PeakA	PeakB	PeakC
AB	19.66	22.28	-	7.22
AC	-	-	6.12	15.40
AD	41.38	-	24.75	40.72
BC	-	13.81	48.69	-
BD	19.32	67.53	-	-
CD	10.46	-	15.87	12.93
A ²	90.70	44.57	117.63	135.81
B ²	8.68	13.88	-	-
C ²	20.20	9.18	36.36	23.12
D ²	94.29	25.73	75.56	62.19

Although ANOVA is sufficient to explain the significance of factors and their interaction terms on the variation of responses, it is still necessary to study the two-dimensional and three-dimensional response graphs to study and understand how the response varies with the slightest changes in input parameter values.

3.3.2. Graphical discussion on various responses.

After ultrasonication pre-treatment, when the extraction temperature reaches 98°C, oil gets collected into the Clevenger oil trap. As seen earlier, the pre-treatment has a major impact on the rate and amount of oil collection.

3.3.2.1. Effects on oil yield.

At a fixed ETime of 75 minutes, varying STemp and Ratio were done to assess STime's impact on Oil Yield. When STemp was held at 40°C, and the ratio increased from 7 to 15, the overall yield decreased by about 0.1 g. The yield showed a tilted reverse-U curve, being relatively higher at lower STime. At 50°C STemp, increasing the ratio from 7 to 15 led to a uniform 0.15 g decrease. However, at 60°C STemp, while there was a 0.1 g decrease, the curve tilted in the opposite direction. This behavior is attributed to shorter sonication times, less water heating, and more efficiency. Synergistically, a higher ratio and ultrasonication time increase oil yield by promoting more cell ruptures. At 50°C STemp and Ratio of 11, increasing extraction time saw near-constant yield at 90 minutes (approx. 0.4 g) and a significant jump from 0.27 g to 0.57 g at 30 minutes, indicating lower sonication time led to lesser volatile losses. The trend continued for ETime increase at minimum STime and Ratio, reaching 0.83 g (from 0.4 g) at 40°C and 0.6 g (from 0.36 g) at 60°C. This highlighted how lower ultrasonication bath temperature limited volatile losses. However, at maximum STime and Ratio, while initially yielding 0.55 g, it decreased to 0.36 g at 60°C due to prolonged extraction. Notably, a faint spicy odor from the cotton plug above the condenser indicated losses through the condenser overhead. Comparing oil yield against ratio, at minimum STime and STemp, the highest yield had the lowest ratio, followed by a decreasing curve as ETime lowered. This indicated a lower ratio, solubilized more volatiles, and facilitated efficient heat and mass transfer, leading to better boiling during longer extractions. Analyzing interactions between factors, when Ratio and ETime were varied, the maximum oil yield was observed at least STime and STemp. The 3D surface showed a decrease as the ratio increased. Elevating ETime at least STime and STemp led to increased yield. The analysis is presented in Figure 3, illustrating Oil Yield with varying interactions.

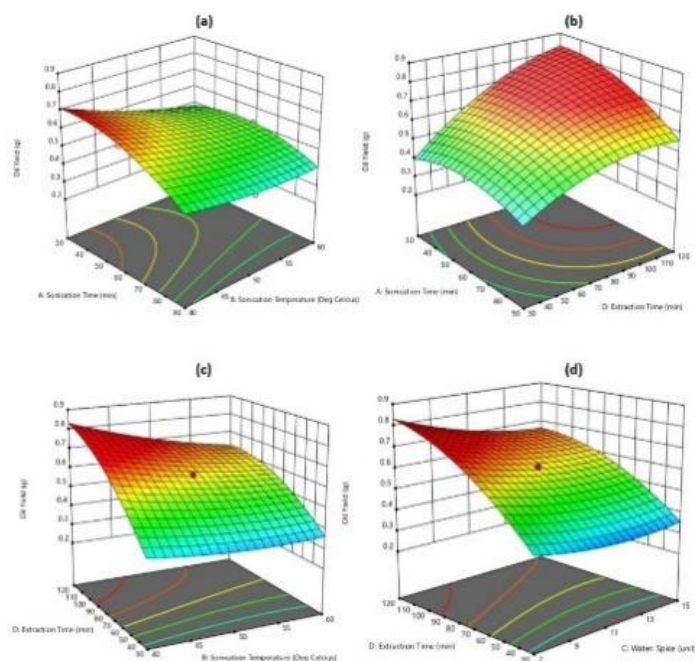


Figure 3. Effect of interaction of Factors on Oil Yield (a) at Ratio 11 and ETime 120min; (b) at STemp 40°C and Ratio 7; (c) at STime 30 min and Ratio 7; (d) at STime 30 min and STemp 40°C.

3.3.2.2. Effects on PeakA content.

Analyzing PeakA's relative area varying factors reveals insights. At constant 50°C STemp and Ratio 11, increasing ETime from 30 to 120 minutes reduces PeakA percentage overall, with slightly higher values at 30 minutes due to Sabinene's volatility. Changing STemp while Ratio and ETime are fixed at 11 and 75 minutes, respectively, alters PeakA differently. At 40°C, PeakA remains nearly constant at 26%, while at 60°C, it increases at lower STime and decreases at higher STime.

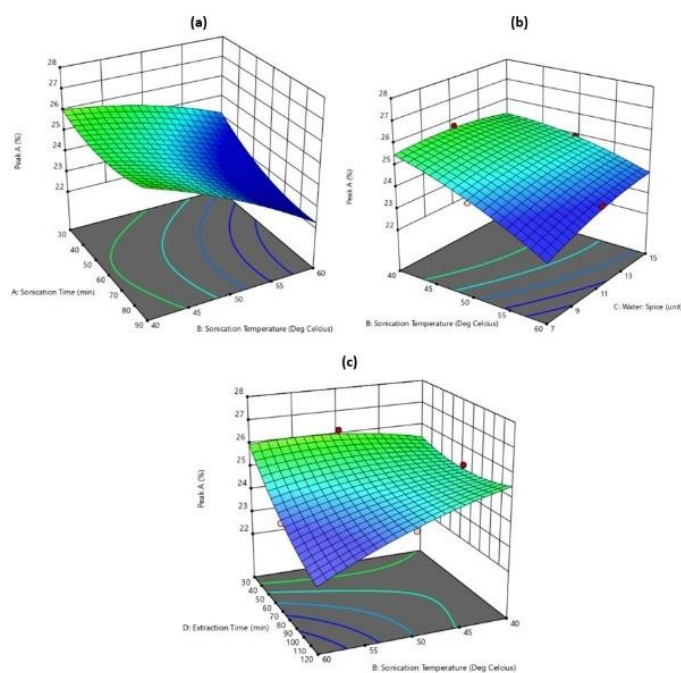


Figure 4. Effect of interaction of Factors on PeakA% (a) at Ratio 7 and ETime 120min; (b) at STime 60min and ETime 120min; (c) at STime 60min and Ratio 7.

Maintaining midpoints (50°C, 75 minutes) and altering ratio shows higher content at 30-minute STime, indicating better extraction at greater Ratios. PeakA% changes with STemp: 40°C remains constant, while at 60°C, it drops from 27% to 24%. Interactions reveal the highest PeakA% at maximum ratio, minimum ETime, minimal STime, and maximum Stemp (Figure 4).

3.3.2.3. Effects on PeakB content.

Analyzing PeakB%, its relationship with STime is squared due to a high F-value. Maintaining constant STemp (50°C) and ratio (11), increasing extraction time reduces PeakB% overall, indicating higher extraction temperatures cause volatile molecule losses, particularly Limonene. Raising the ratio while keeping others constant leads to a dip in PeakB% at 90-minute sonication, implying greater water volume accelerates volatilization during prolonged sonication. Higher STemp generally increases PeakB% across STime, as slightly elevated 60°C temperature aids extraction without substantial losses. STemp maintains a linear relationship with PeakB. With increasing ETime and fixed STime and Ratio, there is a uniform PeakB% decrease, suggesting losses during heating mantle extraction. At Ratio 7, PeakB% drops from 55% (40°C) to 53.5% (60°C), with the trend reversing at Ratio 15. As STime increases, PeakB% uniformly rises, especially at 60°C STemp, indicating longer sonication enhances diffusion.

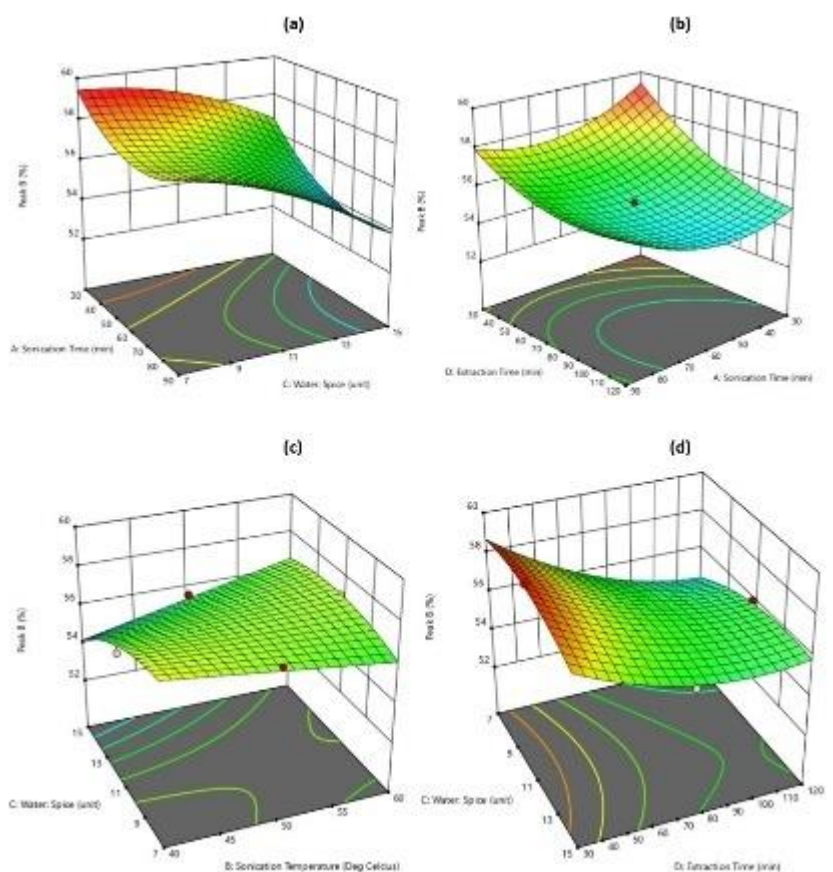


Fig 5. Effect of interaction of Factors on PeakB% (a) at STemp 40°C and STime 30 min; (b) at STemp 40°C and Ratio 7; (c) at STime 60 min and ETime 30 min; (d) at STemp 50°C and STime 30 min.

PeakB% variation with ratio mirrors PeakA%. However, it is not linear but squared, as evidenced by a high F-value. Considering ETime with constant STemp and STime, as the ratio increases, PeakB remains steady at 54% to 120 minutes but drops from 57% to 55.5% at 30

minutes. Complex interactions indicate that a single-factor explanation is insufficient. Elevating STemp slightly increases PeakB% uniformly, with a greater effect at 30 minutes than at 120 minutes ETime, indicating rapid initial extraction of PeakB. Analyzing two-factor interactions, with constant STemp and STime, varying STemp and ETime unveils a peak in PeakB% at minimum ETime for Ratio 7 and 15 at 30-minute STime. The 3D surface descends as ETime rises, indicating milder ultrasonication and shorter extraction enhance PeakB retention. Figure 5 illustrates PeakB content changes due to interactions between STime, STemp, Ratio, and ETime – (a) at STemp 40°C and STime 30 minutes; (b) at STemp 40°C and Ratio 7; (c) at STime 60 minutes and ETime 30 minutes; (d) at STemp 50°C and STime 30 minutes.

3.3.3. Model accuracy checking.

To ensure the validity of the generated models, their accuracy must be assessed. An erroneous model can yield misleading outcomes, while a precise one aids extraction optimization. Figure 6 compares model-predicted values with experimental results. All generated models—Oil Yield, PeakA, PeakB, and PeakC—exhibit clustered actual values around predicted values, confirming the agreement. With high R2 and adjusted R2 values and the alignment of actual and predicted values, these models offer reliable predictions. Thus, the optimization tool proves valuable for further extraction analysis.

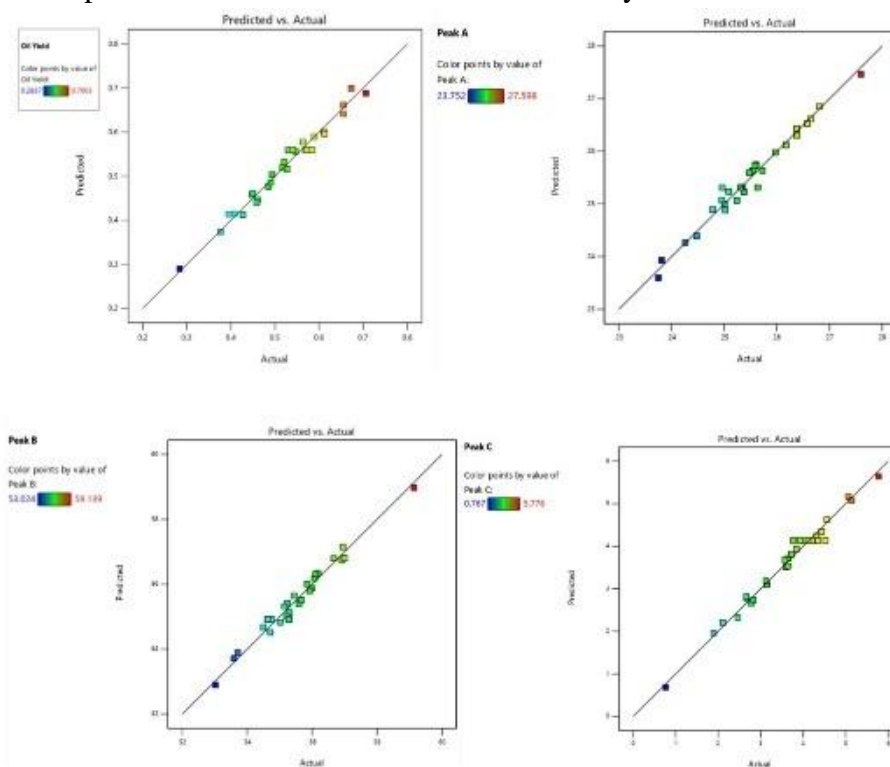


Figure 6. Comparison of predicted and actual values of respective models.

3.3.4. Optimization of extraction parameters and confirmation.

For Radhuni essential oil to be considered of good quality and economical, maximum extraction should be aimed at high content of PeakA and PeakB, which are highly volatile compounds. PeakC content is not that important as it is one of the low boiling compounds. The correlation matrix in Design-Expert shows that oil yield and peak A and peak B percentages are negatively and positively correlated to peak C content, as shown in Figure 7.

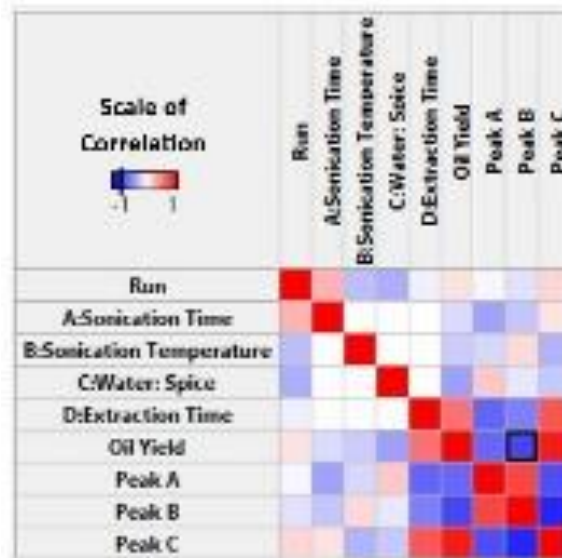


Figure 7. Correlation matrix.

Considering the above-mentioned inferences, the following constraints, as shown in Table 6. were applied for optimization. Based on these constraints fed into the software, 7 solutions of 0.789 desirability were obtained. Comparing the significance of similarities and differences in the Extraction parameters of the solutions, 1 of them was chosen to be experimentally performed to validate the models and repeated thrice. The chosen solution was based on the least extraction time yielding maximum oil yield, which is 30 min of Sonication time at 40°C, the ratio of water to spice at 7, with an extraction time of 54.5 min.

Table 6. Constraints for Optimization.

Name	Goal	Lower Limit	Upper Limit	Importance
A: Sonication Time	minimize	30	90	+++++
B: Sonication Temperature	is in range	40	60	+++
C: Water: Spice	minimize	7	15	++++
D: Extraction Time	minimize	30	120	+++++
Oil Yield	maximize	0.2837	0.7063	+++++
Peak A	maximize	23.752	27.598	+++
Peak B	maximize	53.024	59.139	+++
Peak C	is in range	0.767	5.776	++

To validate the model, the responses of the solutions obtained were averaged (Data Mean) and then compared with the predicted values given by the model, as shown in Table 7.

Table 7. Validation of averaged solutions.

Analysis	Predicted Mean	Predicted Median	Standard Deviation	95% PI low	Data Mean	95% PI high
Oil Yield	0.575766	0.575766	0.0192607	0.51932	0.6247	0.632212
Peak A	25.5376	25.5376	0.188629	24.9094	25.087	26.1658
Peak B	57.4026	57.4026	0.289069	56.4398	56.81	58.3653
Peak C	3.6123	3.6123	0.207464	2.92137	3.3	4.30322

Table 7. illustrates that the actual value of optimized response variables lies within a 95% Probability Interval of the value predicted by the generated models. As the results obtained are accurate, it can be concluded that the statistical model obtained using the 4-factor Box-Behnken design is valid and reliable and thus can be used to assess the optimal extraction conditions for the UAHD Technique of Radhuni essential oil.

3.4. Moisture of fruit and extraction efficiency.

To make any production process feasible and economically viable for essential oils, the two things to note are its oil yield and the quality of oil. From the same spice packet, the moisture or loss content analysis was drawn and concluded in Table 8.

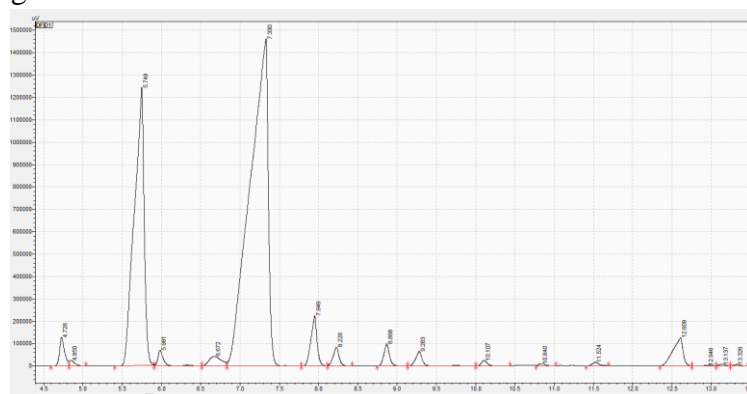
Table 8. Moisture content and extraction efficiency comparison.

Method	Results (on a dry basis of spice)	
Toluene Displacement Method (ISO 939:2021)	6.12% (pure moisture content)	
Hot Air Oven Drying Method (AOAC 930.15)	8.04% (both moisture and volatile loss)	8.075% average
Infrared Moisture Analysis (ISO 248:2019)	8.11% (both moisture and volatile loss)	
Total Essential oil in fruit		1.955% (g/100g) or 2.287% (ml/100g)
	EO extracted (%) (ml/100g)	Extraction efficiency (%)
HD	0.78	34.10
UAHD	1.64	72.09

The official method for determining moisture content in spices, the toluene displacement method, revealed 6.12% moisture during analysis. Employing two other drying methods, the overall loss percentage, including moisture and volatile oil, averaged 8.075%. Consequently, the volatile oil in the fruit was found to be 2.287%. The toluene method was impractical for daily moisture estimation during precise spice weighing for RSM due to toxic solvent use and high sample quantity requirement (20 g+). Instead, the IR drying method was adopted, requiring only 10-12 minutes and 1 g of sample. Minimal volatile oil loss from sealed packs validated this method, with all percentages presented on a dry basis. Following UAHD optimization and point confirmation, conventional HD and UAHD extraction efficiency was calculated. UAHD conditions were previously stated, while HD extraction used 45 g (dry basis), W-S Ratio 11, and 4.5-hour ETime, yielding 0.302 g (0.78%). Thus, extraction efficiencies were 34.10% for HD and 72.09% for UAHD. UAHD displayed superior efficiency and less oil wastage. HD and optimized UAHD processes' essential oil extracts were utilized for further analysis.

3.5. GC and GC-MS analysis of the oils.

As per the developed program mentioned in materials and methods, all 29 Standard runs of RSM were processed through it. A proper resolution was obtained in all the runs, and the Retention time was more or less constant up to ± 0.1 min for the chosen 25 peaks, as shown in Figure 8 and Figure 9.



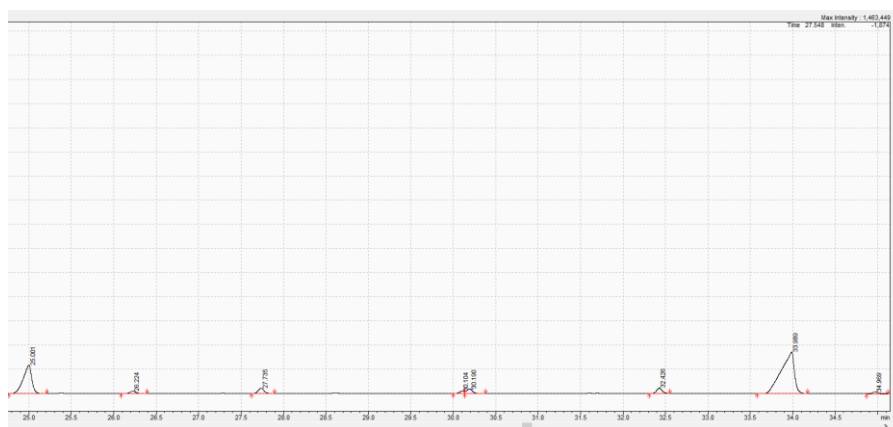


Figure 9. Gas chromatogram of Radhuni essential oil (UAHD) from 25-35 min.

While 0.2 μ L of pure essential oil was injected into the GC, optimization of RSM batches was based on Relative Area % to account for variations in injection volume. Relative area % offers a comparative content result, reflecting the inverse relationship between increasing levels of one compound and the decrease of another. Although it does not provide the true % chemical composition, it offers a relative chemical composition assessment. The GC program ran for 46 minutes, but significant peaks ceased after 35 minutes; thus, chromatograms show results up to 35 minutes. Essential oil samples were then subjected to GC-MS analysis at the Chemical Engineering Department of ICT, Mumbai, using a similar method with a different column (DB5-ms for GC-MS and Rtx-5 for GC).

Table 9. Comparison of relative composition of essential oil.

Retention Time (min)	Chemical Name	Relative composition (%)		CAS Number
		UAHD	HD	
4.728	α -Thujene	1.322	1.160	2867-05-2
4.850	α -Pinene	0.279	0.359	80-56-8
5.749	Sabinene	25.087	23.160	3387-41-5
5.981	α -Phellandrene	0.848	0.710	555-10-2
6.672	β -Myrcene	1.094	0.901	123-35-3
7.330	D-Limonene	56.810	54.154	5989-27-5
7.949	γ -Terpinene	2.294	2.712	99-85-4
8.228	α -Terpinene	1.025	1.162	99-84-3
8.868	(Z)-Sabinene hydrate	1.159	1.127	15537-55-0
9.283	(Z)- β -Terpinene	0.991	0.952	586-62-9
10.107	cis-2-Menthenol	0.332	0.404	29803-82-5
10.840	trans-2-Menthenol	0.167	0.124	29803-81-4
11.524	Terpinen-4-ol	0.262	0.277	562-74-3
12.609	Methyl 5-methyl hexanoate	2.061	2.623	2177-83-5
12.946	Methyl heptanoate	0.054	0.216	106-73-0
13.137	Methyl decanoate	0.097	0.319	110-42-9
13.326	Methyl cyclopentaneundecanoate	0.097	0.121	25779-85-5
25.001	cis-4-Hepten-2-one	1.646	2.245	36678-43-0
26.224	D-Germacrene	0.117	0.154	23986-74-5
27.735	β -Caryophyllene	0.259	0.307	87-44-5
30.104	cis- α -Bisabolene	0.095	0.283	29837-07-8
30.190	Aromadendrene	0.220	0.448	109119-91-7
32.426	α -Humulene	0.272	0.710	6753-98-6
33.989	(Z)-Ligustilide	3.300	5.149	81944-09-4
34.969	α -Farnesene	0.112	0.223	502-61-4
	Total	100.000	100.000	

Table 9 identifies 25 desired compounds in essential oil using the NIST library and literature. As per NIST guidelines, the Match Factor (SI) and Reverse Match Factor (RSI) aided careful identification, considering compounds with SI or RSI over 900 as best matches.

Sabinene and Limonene content was higher in UAHD (25.087%, 56.81%, respectively) compared to HD (23.16%, 54.145%, respectively). Conversely, Ligustilide content was lower in UAHD (3.3%) than in HD (5.149%). This clear difference indicates that longer extraction times brought out larger amounts of less volatile components, while highly volatile ones were lost during longer distillation. These three compounds, PeakA, PeakB, and PeakC (representing Sabinene, Limonene, and Ligustilide), are of particular interest in RSM. Additionally, UAHD contains 10.087% terpenes and 4.716% alcohols, ketones, and esters, while HD has 11.208% terpenes and 6.329% alcohols, ketones, and esters.

3.6. Quality characterization of HD and UAHD.

Having large quantities of monoterpenes (almost 80-85%) in the oil, the oil becomes very sensitive to light and heat, sometimes degrading the esters to acids even after extraction. So, to preserve the quality of oil, it is stored in dark amber bottles under refrigerated conditions.

3.7. Antioxidant activity comparison using DPPH.

When free-radical DPPH interacts with an odd electron, the greatest absorption takes place at 517 nm (purple color) [20-24]. A free-radical scavenger antioxidant present in the essential oil extracts reacts to DPPH to form DPPH-H, which has a lower absorbance than DPPH because of the lower amount of hydrogen. Compared to the DPPH state, this radical version causes decolorization (produces a yellow hue) as the number of electrons collected increases. According to the method stated before, the DPPH radical scavenging activity calculations are shown in the following Table 10 and Table 11. The Blank absorbance at 517 nm was 1.1099 throughout the test. With respect to this blank, % inhibition was calculated further (Figure 10).

Table 10. The calculation for IC₅₀ value for UAHD and HD oils.

Oil (ml)	Methanol (ml)	conc (µL/mL)	UAHD			HD		
			Conc (µg/mL)	Absorbance	Inhibition %	Conc (µg/mL)	Absorbance	Inhibition %
0.4	3.6	100	84750	0.2276	79.49365	86210	0.3275	70.49284
0.35	3.65	87.5	74156.25	0.2948	73.43905	75433.75	0.4212	62.05064
0.3	3.7	75	63562.5	0.3621	67.37544	64657.5	0.5367	51.64429
0.25	3.75	62.5	52968.75	0.4303	61.23074	53881.25	0.6277	43.44536
0.2	3.8	50	42375	0.4973	55.19416	43105	0.7005	36.88621
0.15	3.85	37.5	31781.25	0.5638	49.20263	32328.75	0.7852	29.25489
0.1	3.9	25	21187.5	0.6458	41.81458	21552.5	0.8541	23.04712
0.05	3.95	12.5	10593.75	0.7111	35.93116	10776.25	0.9235	16.79431
0.25	3.975	6.25	5296.875	0.7946	28.40796	5388.125	0.9929	10.54149
			IC ₅₀ Value = 35417 µg/mL			IC ₅₀ Value = 60086.3 µg/mL		

Table 11. Calculation for IC₅₀ value for ascorbic acid.

Ascorbic Acid (µg/mL)	Absorbance	%inhibition
50	0.1145	89.68376
45	0.1913	82.76421
40	0.2762	75.11488
35	0.3842	65.38427
30	0.4504	59.41977
25	0.5355	51.75241

Ascorbic Acid (µg/mL)	Absorbance	%inhibition
20	0.6373	42.58041
15	0.7106	35.97621

IC₅₀ Value = 24.258 µg/mL

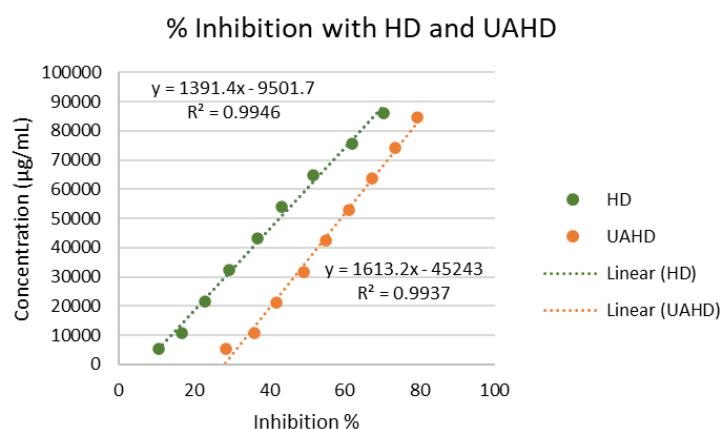


Figure 10. Graphical plot of oil concentration vs inhibition %.

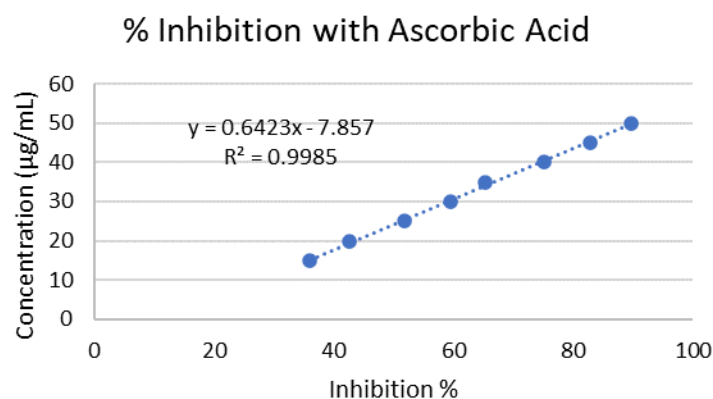


Figure 11. Graphical plot of ascorbic acid concentration vs inhibition %.

With the obtained data, inhibition % was plotted along the various concentrations of UAHD, HD oils, and ascorbic acid, as shown in Figures 10 and 11. From these graphs, the linear regression curve was plotted, and IC₅₀ values were calculated. It was seen that IC₅₀ of UAHD oil (35417 µg/mL) was more than that of HD oil (60068.3 µg/mL), whereas that of a well-known natural antioxidant Ascorbic acid was (24.258 µg/mL) more than 1000 times than the oils (Figure 11).

3.8. Summary of all results.

All the conclusions drawn from each characterization of HD and UAHD oil are summarized in Table 12. as mentioned below.

Table 12. UAHD and HD comparative results summary.

	UAHD	HD
Oil yield	1.64%	0.78%
Composition of 3 important compounds	Sabinene- 25.087%, Limonene- 56.81%, Ligustilide- 3.3%	Sabinene- 23.16%, Limonene- 54.154%, Ligustilide- 5.149%
Extraction Efficiency	83.88%	39.83%
Appearance	Pale yellow, transparent, non-viscous, very diffusive	Slightly more yellowish, transparent, non-viscous, very diffusive
Odour Profile	spicy, more citrus, more terpenic, and woody	was more spicy, less citrus, terpenic, and more woody

	UAHD	HD
Grease Test	No impression after 10min	Negligibly visible taint on rims
Colour (AE)	3.077	4.066
Solubility	Soluble in acetone, hexane, IPA, methanol, 90% ethanol, DPG, PG; Insoluble in water, 80% methanol; Partially soluble with bubbles and cloud formation in 70-80% ethanol	Soluble in acetone, hexane, IPA, methanol, 90% ethanol, DPG, PG; Insoluble in water, 80% methanol; Partially soluble with bubbles and cloud formation in 70-80% ethanol
Specific gravity	0.8475	0.8621
Refractive index	1.4718	1.4689
Acid value	1.2446 mg KOH/g oil	1.8673 mg KOH/g oil
Saponification value	36.91 mg KOH/g oil	36.56 mg KOH/g oil
Ester Value	35.6653 mg KOH/g oil	34.6926 mg KOH/g oil
Antioxidant value (IC₅₀)	35417 µg/mL	60068.3 µg/mL

4. Conclusions

From the initial trials, we concluded that grinding the spice results in more oil yield due to mechanical rupturing of oil sacs. The optimization of UAHD treatment led to the finding of a successfully higher yield of essential oil compared to the HD technique. At the same time, valuable water-ethanol soluble extractables could be isolated from the DIFE process. The physical and chemical Characteristics of the essential oils showed a comparison and slight difference between the 2 techniques, proving UAHD oil is of better quality. The oil showed good antioxidant properties, which enriches food products. Furthermore, it can be used in liquid spice mix formulations for flavoring. The spent spice is good fuel to help achieve a circular economy for the raw materials.

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Conflicts of Interest

The authors declare no conflict of interest.

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