

Ultrasound-assisted extraction and RP-HPLC quantification of β -caryophyllene in plant essential oils: Separation efficiency and insecticidal activity

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ABSTRACT

This study details the development of a method for separating essential oils using ultrasound-assisted extraction (UAE) and quantifying β -caryophyllene in 14 plant samples via reverse phase-high performance liquid chromatography (RP-HPLC). Extraction efficiencies of methods like hydrodistillation, Soxhlet extraction, and ultrasonic extraction were compared, with n-hexane, methanol, and ethyl acetate as solvents. Notably, UAE with n-hexane showed the highest β -caryophyllene yield in HPLC analysis. Separation was achieved within 20 min using isocratic elution on a C-18 reverse-phase column, with a mobile phase of acetonitrile and water (70:30) at room temperature and detection at 210 nm via diode array. The method showed excellent linearity (0.1–5 $\mu\text{g/mL}$), with LOD and LOQ values of 0.02 and 0.07 $\mu\text{g/mL}$, respectively, achieving high accuracy (recovery rate of 102.1%) and precision (RSD 0.45%). Additionally, the insecticidal potential of β -caryophyllene, isolated from *Hyptis suaveolens* essential oil, was tested against two storage pests, *Sitophilus granarius* and *Coryca cephalonica* larvae, demonstrating significant insecticidal activity. This suggests that β -caryophyllene could serve as an effective natural insecticide.

1. Introduction

The global demand for essential oils is significantly increasing due to their environmentally friendly attributes and fascinating physicochemical characteristics [1]. Essential oils are natural volatile compounds extracted from plants, valued for their aromatic, therapeutic, and antimicrobial (bactericides, fungicides, insecticides, pesticides, and virucides) properties. They are widely used in medicine, cosmetics, food, and aromatherapy for health and wellness benefits [2–4]. β -caryophyllene, or trans-caryophyllene, chemically known as (1R, 4E, 9S)–4,11,11-trimethyl-8-methylidenebicyclo[7.2.0] undec-4-ene, is a bicyclic sesquiterpene widely found in various herbs and spices [5]. The primary constituent found in both volatile and non-volatile essential oils is widely acknowledged [6]. This bioactive compound boasts a myriad of pharmacological benefits, encompassing anti-inflammatory,

anti-tumoral, antibacterial, anti-edema, anti-allergic, antimicrobial, antifungal, and phage-repellent effects [7–9]. For example, research highlights significant anti-inflammatory activity against neuroblastoma and lymphoma cells, demonstrated by β -caryophyllene extracted from *Cordia verbenacea* and *Aegle marmelos* [10,11]. The anti-tumor effects of β -caryophyllene derived from *Commiphora gileadensis* have also been documented [5]. Recent investigations indicate the potential of β -caryophyllene emitted from genetically engineered Arabidopsis plants to effectively combat *Diaphorina citri*, a vector of *Candidatus Liberibacter*, thus safeguarding citrus plants from yellow shoot disease [12]. Moreover, β -caryophyllene exhibits neuroprotective properties, showing promise in treating various nervous system disorders such as anxiety, depression, convulsions, and Alzheimer's disease, primarily through its interaction with cannabinoid receptors CB2R [6]. While extensive research has been conducted on the biological activities of

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β -caryophyllene for its diverse potential applications in various pathological conditions, studies pertaining to its pesticidal properties remain lacking. It's noteworthy that all investigations on this molecule are preclinical (in-vivo, in-vitro, or in animal models), necessitating further exploration of its toxicological and pharmacological characteristics through clinical trials involving humans, animals, and insects [13]. Consequently, considering the myriad biological properties of β -caryophyllene, there is a need to explore fast identification and quantification techniques in various native spices and herbs.

Various conventional methods exist for extracting essential oils, each tailored to different plant types and with unique applications. Common techniques include hydrodistillation, steam distillation, solvent extraction, and cold pressing [1,3]. However, these methods often come with drawbacks such as high energy consumption, thermal damage risks, solvent residues, and limited applicability to certain plants. Non-conventional extraction methods offer innovative alternatives to traditional techniques. Supercritical fluid extraction, typically utilizing CO_2 , operates at moderate temperatures and pressures, resulting in high-purity oils [14]. Microwave-assisted extraction and solvent-free microwave extraction utilize microwave energy for efficient oil extraction without solvents. Ultrasound-assisted extraction (UAE) employs ultrasonic waves to enhance extraction efficiency [3]. Subcritical extraction with CO_2 or H_2 utilizes lower pressure and temperature for gentle oil extraction [14]. Researchers have explored various extraction methods to improve efficiency and yield. Ugarte et al. achieved enhanced essential oil yields from basil and epazote plants using microwave-assisted extraction [15]. Yousefi et al. demonstrated the effectiveness and rapidity of supercritical fluid extraction without toxic organic solvents at moderate temperatures [16]. Elyemni et al. compared microwave-assisted hydrodistillation with traditional hydrodistillation, finding the former yielded higher extraction rates, suggesting its suitability for industrial-scale production [17]. UAE extraction is gaining attraction as a green alternative to conventional methods due to its ability to generate environmentally contaminant-free products [18]. This method utilizes ultrasound to induce cavitation, promoting the release of bioactive compounds or oils from cell matrices [19]. Compared to other methods, UAE offers advantages such as cost-effectiveness, reduced time and energy requirements, lower solvent consumption, lower extraction temperatures, and preservation of oil quality [20]. Researchers have optimized ultrasonic-assisted extraction for specific compounds, such as eugenol from basil leaves, achieving efficient extraction within minutes [21]. Additionally, ultrasound-assisted hydrodistillation has been shown to enhance the efficiency of traditional hydrodistillation processes for extracting essential oils from celery seeds [22]. These methods generally reduce extraction time and solvent use, but may require specialized equipment and higher initial costs. Based on studies it is found that essential oils including β -caryophyllene was generally extracted by traditional methods such as steam distillation or hydrodistillation that takes longer time for extraction. Therefore, some advanced innovative methods need to be implemented for rapid extraction of essential oil without affecting the quality and content of compounds. UAE is such method that require less time for extracting phytoconstituents. Thereby, this can be implemented for extraction of essential oil.

Gas chromatography (GC), GC-mass spectrometry [23], high performance liquid chromatography (HPLC) was mostly used analysis of β -caryophyllene in several plant samples. GC requires high temperatures, which can degrade heat-sensitive compounds. GC-MS is essential for their analysis, offering high sensitivity and structural identification of several volatile essential oils. Its superior separation makes it ideal for complex mixtures [24–26]. Though less common, high-performance liquid chromatography (HPLC) can analyze volatile compounds, especially thermally unstable ones or derivatized forms. HPLC's versatility allows for a range of detection methods, including UV and diode array detection, enhancing its sensitivity and selectivity. Additionally, HPLC requires less sample preparation and can handle non-volatile

compounds, making it ideal for analyzing essential oils and plant extracts [27]. Park et al. developed a GC method for quantifying β -caryophyllene in clove extract. Their method demonstrated high linearity and better accuracy with corresponding values for relative standard deviation (RSD), limit of detection (LOD), and limit of quantification (LOQ) [28]. Lucca et al. outlined the validation of a novel, solvent-free GC method in headspace mode coupled with mass spectrometry to assess skin impregnation or retention of β -caryophyllene from crude copaiba oil and its nanoemulsion. Their results indicated high linearity, precision (RSD < 15 %), and accuracy (recovery > 90 %) [29]. Gomes et al. developed and validated a HPLC method for quantifying α -humulene and β -caryophyllene in *Cordia verbenacea* essential oil and pharmaceutical formulations using the Box-Behnken model. They discovered that the concentration of β -caryophyllene in *Cordia verbenacea* was approximately four times higher than humulene [26]. A similar study conducted on *Copaiba oleoresin* revealed chromatographic peaks with good symmetry and resolution, with a linearity range, suggesting HPLC as a viable alternative to GC for β -caryophyllene quantification [27]. The limitations of these studies include potential variability in sample preparation, limited scope regarding the range of β -caryophyllene concentrations tested, possible matrix effects not accounted for, and a lack of inter-laboratory validation.

The present study aimed to develop and optimize a rapid and precise RP-HPLC-DAD method to quantify β -caryophyllene in 14 plant samples, aligning with ICH guidelines. The goal was to ensure reliability, key validation parameters, including linearity, accuracy, precision, LOD, LOQ, RSD, and recovery, were rigorously assessed. The research also compared three extraction methods, hydrodistillation, Soxhlet extraction, and ultrasonic extraction to identify the most efficient technique for obtaining high-quality essential oils. Additionally, the insecticidal activity of β -caryophyllene against two stored-grain pests was investigated, suggesting potential use as a natural insecticide. The study addressed a gap in quantifying β -caryophyllene from plant samples in the Chhattisgarh region of India, where its pharmacological potential remains largely unexplored.

2. Materials and methods

2.1. Materials

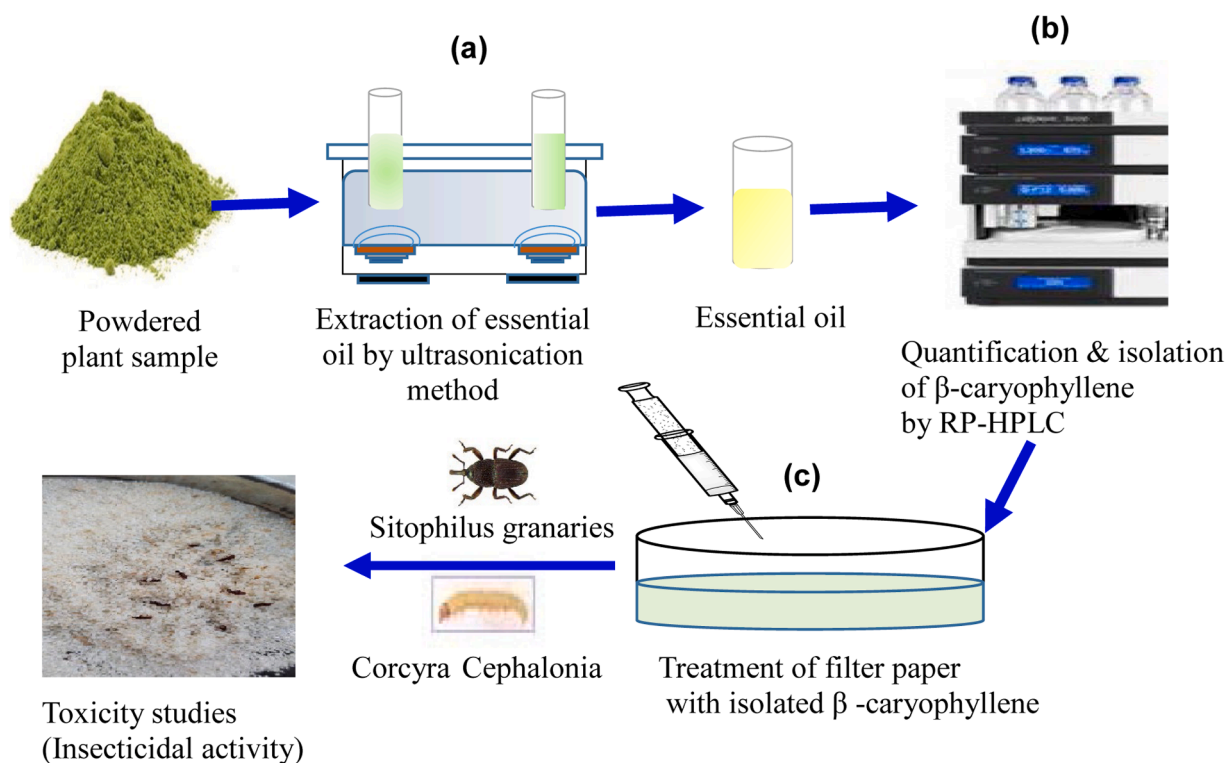
β -caryophyllene (>96 % purity) was purchased from Sigma Aldrich PVT. LTD. (India). Solvent HPLC grade Acetonitrile, Water, and Methanol were purchased from J. T. Baker (Avantor Performance Materials, India Ltd.), and n-hexane and ethyl acetate were purchased from Hi-Media PVT. LTD. (Mumbai India)

2.2. Plant sample collection

Different plant samples were collected from different regions of Chhattisgarh (India). Leaves samples of curry leaves (*Murraya koenigii*), lemon (*Citrus lemon*), sweet basil (*Ocimum basilicum*), eucalyptus (*Eucalyptus citriodora*), holy basil (*Ocimum tenuiflorum*), Indian bay leaf (*Cinnamomum tamala*), wild leaf basil (*Hyptis suaveolens*), green leaf basil or rama tulsi (*Ocimum sanctum*), satinwood (*Chloroxylon swietenia*), buds of clove (*Syzygium aromaticum*), bark of cinnamon (*Cinnamomum verum*) and seed of coriander (*Coriandrum sativum*), cumin (*Cuminum cyminum*), Ajwain (*Trachyspermum ammi*) were collected in polyethylene bags.

2.3. Extraction of essential oils

After washing and drying, plant materials were first fine-powdered using mortar-pestle. The essential oils were then extracted by three different methods termed hydro-distillation (using Clevenger apparatus), Soxhlet extraction, and UAE methods. In hydro distillation method 10 g of powdered samples were mixed with (water and different organic solvents; methanol, n-hexane and ethyl acetate were taken in



Scheme 1. (a) demonstrated the sample preparation for extraction of essential oils; (b) HPLC analysis; and (c) assessment of insecticidal activity of β -caryophyllene.

equal volumes (100 mL) and boiled in a round bottom flask for 3 h to get a pale-yellow color oil as per methods described earlier with some modifications. The oils were collected in the amber-reagent bottle to store at 4 °C for further use [30]. However, in Soxhlet method, 10 g of fine plant powder was mixed with 20 mL of different organic solvents (n-hexane, methanol, and ethyl acetate) in a round bottom flask attached to the Soxhlet extractor. After 5 h of extraction, the upper layer of organic solvent was evaporated by a rotary evaporator to recover essential oils [31]. In contrast, ultra-sonication extraction was performed in an ultrasonic bath (PCI Analytics Pvt. Ltd., Mumbai, India) of 44 kHz ultrasonic frequency with multiple transducers. A 10 g fine powder of tested plant samples was mixed with 20 mL of n-hexane/methanol or ethyl acetate followed by sonication for 30 min at 25 °C [30]. The organic layer was then transferred into a separating funnel and it was treated with 20 mL of 1 % NaCl solution for recovering essential oils. The obtained extract with essential oil was sonicated three times with the addition of 50 mL of solvent each time to get a yield of three fractions of essential oil. Schematic diagram for the sample preparation for the extraction of essential oils is illustrated in Scheme 1(a). The percentage yield of essential oil is determined by using following formula [23]:

$$\% \text{ Yield} = \frac{\text{Weight of extract with container} - \text{Weight of container}}{\text{Sample weight taken for extraction}} \times 100$$

2.4. HPLC analysis of β -caryophyllene

The stock of pure β -caryophyllene (100 $\mu\text{g/mL}$) was prepared in HPLC grade acetonitrile. Different amount of pure β -caryophyllene ranging 0.1, 0.5, 1, 2, 3, 4, 5 $\mu\text{g/mL}$ were prepared in amber colored glass bottle at 4 °C to prevent sunlight exposure. The quantitative analysis of pure β -caryophyllene used as standard and tested 14 plant essential oil containing β -caryophyllenes were performed using UHPLC-DAD (Ultimate 3000, Thermo Fisher Scientific, Madison, USA). For this, C-18 reverse phase column (Hypersil GOLD™, 100 $\times$ 4.6 mm $\times$ 5 μm ;

Thermo Fisher Scientific, Madison, USA) was used for the quantification. Different parameters of RP-HPLC-DAD conditions were also optimized for the separation of β -caryophyllene from plant samples. Separation and identification of pure β -caryophyllene were carried out using isocratic elution by taking solvent acetonitrile and water in 70: 30 ratios. The flow rate was maintained at 1 mL min⁻¹ and wavelength was 210 nm, shown in scheme 1(b).

2.5. FTIR analysis of β -caryophyllene derived compound

The functional group identification of isolated β -caryophyllene were assessed by FT-IR (Bruker Alpha Eco-ATR (Bruker India Scientific Pvt. Ltd.) equipped with zinc selenide (ZnSe) crystal cell in the wavenumber range from 4000 to 400 cm⁻¹. For FTIR characterization 20 $\mu\text{g/mL}$ pure β -caryophyllene and same concentration of isolated compound were prepared in acetonitrile solvent. The FTIR spectra of standard sample of β -caryophyllene and isolated β -caryophyllene from plant sample was recorded.

2.6. Validation of HPLC method

Validation of optimized analytical method for the quantification of β -caryophyllene was performed according to the United States Pharmacopeia (USP) and International Conference on Harmonization [27]. For this, various parameters of validation such as linearity, precision, accuracy, the limit of detection (LOD), limit of quantification (LOQ), repeatability and recovery of method proposed were studied.

2.6.1. Linearity range

Linearity was evaluated by analyzing different concentration of standard solutions of β -caryophyllene in triplicate with a concentration range from 0.1, 0.5, 1, 2, 3, 4, 5 $\mu\text{g/mL}$. The calibration curve was obtained by plotting peak area with their corresponding concentrations, and statistical analysis was done by linear regression analysis by using least squares method in Microsoft Excel software. Correlation coefficient of value near to the 1 indicated the excellent linearity of different

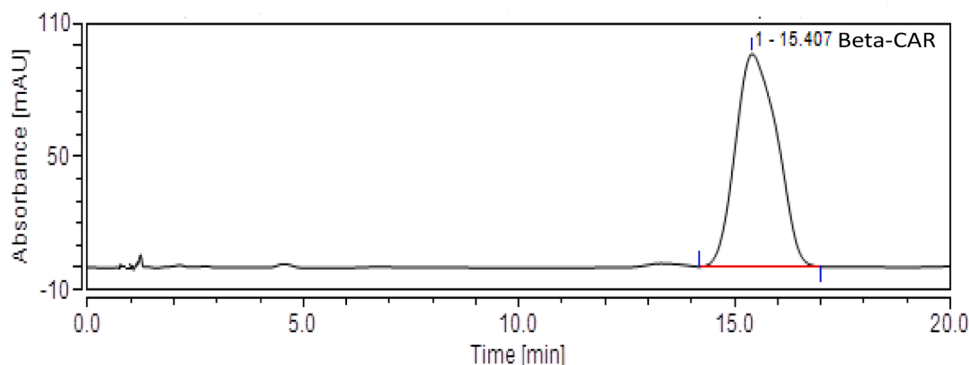


Fig. 1. Chromatographic separation of β -caryophyllene using an isocratic mobile phase of acetonitrile and water (70:30 v/v), with a retention time of 15.4 min and detection at a wavelength of 210 nm, employing a flow rate of 1 mL/min.

concentration of analyte in the calibration curve [26].

2.6.2. Precision

Precision was assessed by 5 replicate measurements. To determine the precision 5 replicates of 1 $\mu\text{g/mL}$ of standard β -caryophyllene solution were successively determined and results were indicated in terms of relative standard deviation (% RSD). RSD % was obtained smaller than 2 % showing better precision [26].

2.6.3. Accuracy

The accuracy of proposed method was evaluated in terms of percentage recovery by spiking known concentration of β -caryophyllene standard solution into blank or real sample of any one of essential oil i.e. *Hyptis suaveolens* [26]. The recovery percentage was calculated by using the formula as below:

$$\text{Recovery \%} = \frac{\text{concentration after spiked} - \text{concentration before spiked}}{\text{concentration of spiked}} \times 100$$

2.6.4. Specificity

The selectivity or specificity of the proposed method was determined by spiking different foreign chemical substances that might present in real samples. For this, different substances, diluents, other solvents were injected into the HPLC, if there were no other peaks in chromatogram at retention times of target analyte indicated that the proposed illustrating the specificity for the measurement of β -caryophyllene [26].

2.6.5. Limit of detection (LOD)

The LOD of proposed method was determined by calculating the ratio of three times of standard deviation of intercept to average slope (s) obtained from three calibration curve [26]. Formula to calculate LOD is given below:

$$\text{LOD} = \frac{3 \times \text{SD}}{s}$$

2.6.6. Limit of quantification (LOQ)

The LOQ of proposed method was evaluated by calculating the ratio of ten times of standard deviation of intercept to average slope (s) obtained from three calibration curve [26]. Formula to calculate LOQ is given below:

$$\text{LOQ} = \frac{10 \times \text{SD}}{s}$$

2.7. Procedure for assessment of insecticidal activity of isolated β -caryophyllene

For assessment of insecticidal activity of isolated β -caryophyllene

from different plant samples, two grain pest *Sitophilus granarius* and larvae of *Corcyra cephalonica* were reared in five containers covered with muslin cloth for proper aeration at $25 \pm 3^\circ\text{C}$ with relative humidity of 65–70 % using rava (semolina) diet purchased from the local market, demonstrated in scheme 1(c). These pests are reared male and female in each container and 5 days to permit oviposition. The insecticidal study was done with these pests [32]. For this, different amount (100 - 500 $\mu\text{g mL}^{-1}$) of isolated β -caryophyllene were sprayed on Whatman filter paper No. 1 and placed in semolina diet containing Petridis after 20 min. About 25 *S. granarius* and *C. cephalonica* larvae were transferred on the plate covered with muslin cloth for assessment of pest mortality at different time intervals of 1, 6, 12, 24 and 48 h of incubation [32]. The experiment was set up in 5 different replicates. Mortality rate was calculated using Abbot's formula [33]:

$$\text{Mortality \%} = \frac{\text{number of dead insects}}{\text{total number of insects taken}} \times 100$$

3. Results and discussion

3.1. Optimization of experimental condition for measurement of β -caryophyllene

The optimization of experimental conditions for measuring β -caryophyllene in plant-extracted samples via HPLC is vital for accurate and insightful analysis. The optimization of parameters such as solvent composition, column type, and detection wavelength ensures optimal separation and detection of β -caryophyllene amidst complex plant matrices. This optimization maximizes sensitivity, specificity, and reproducibility, crucial for robust data interpretation and comparison across samples.

Gradient and isocratic elution in HPLC play vital roles in optimizing peak resolution and retention times. Gradient elution enhances resolution but may co-elute compounds, while isocratic elution simplifies analysis but can lead to longer retention times [27]. The proper selection ensures accurate and efficient chromatographic separation, crucial for reliable results. In the present work, isocratic elution was used for separation of β -caryophyllene using fixed solvent acetonitrile: water ratio (70:30). Further, the flow rate in high-performance liquid chromatography (HPLC) is crucial as it determines the speed and efficiency of analyte separation within the chromatographic column, impacting resolution and analysis time [27]. For optimizing the flow rate in HPLC, the method was executed at flow rates of 0.5, 0.75, and 1.0 mL/min (Fig. S1), revealing that at 1 mL/min, a distinct and sharp chromatographic peak with well-defined characteristics emerged at 15.4 min of retention time, leading to its selection for subsequent determinations. The improved performance observed at a flow rate of 1 mL/min in HPLC could be attributed to the balance between adequate elution strength and optimal efficiency, facilitating optimal analyte separation.

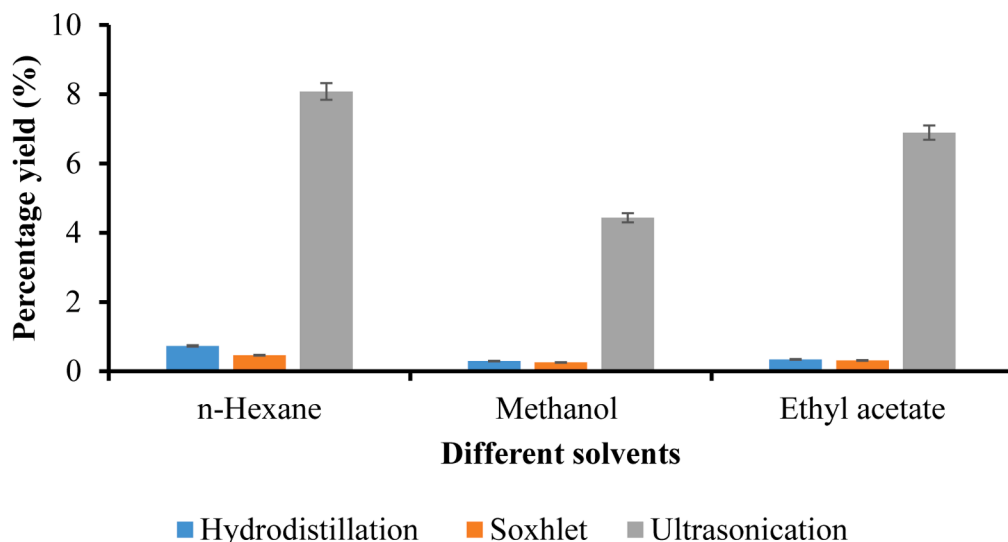


Fig. 2. Percentage yield of essential oils using different solvents and extraction methods.

The mobile phase (solvent) ratio in HPLC significantly influences separation by affecting analyte retention, selectivity, and peak shape. By adjusting the composition of the mobile phase, chemists can fine-tune interactions between the analyte and stationary phase, optimizing separation conditions for enhanced resolution and accuracy. The optimization of β -caryophyllene separation in HPLC involved varying the mobile phase solvents, acetonitrile and water, chosen for their ability to maintain a linear baseline and symmetrical chromatographic signals due to their lower cutoff value [27]. Various ratios of acetonitrile to water (90:10, 70:30, and 60:40) were tested, revealing that the 70:30 ratio produced a sharp peak at 15.4 min of retention time, while the 90:10 and 60:40 ratios resulted in coelution with other compounds, thus prompting their exclusion for further studies (Fig S2). Previous reports noted the use of methanol and methyl nitrile, but they yielded poor separation with asymmetrical chromatography [27].

The selection of wavelengths for DAD detector in HPLC ensures precise identification and quantification of target compounds. The optimal wavelengths enhance specificity, sensitivity, and peak resolution, crucial for accurate analysis. This minimizes interference, maximizes signal-to-noise ratio, and ensures reliable detection even in complex samples, optimizing analytical performance. Various wavelengths (210 nm, 215 nm, and 220 nm) were assessed for the measurement of β -caryophyllene absorption. Notably, the 210 nm

wavelength demonstrated superior sensitivity compared to 215 nm and 220 nm. The findings indicated that β -caryophyllene peak resolution was optimized under isocratic flow conditions using a 70:30 mixture of acetonitrile and water, observed at 15.4 min of retention time, specifically at the 210 nm wavelength, with a flow rate of 1 mL/min, as illustrated in Fig. 1.

3.2. Optimization of extraction methods for separation of essential oils

The determination of the percentage yield of essential oils using solvents ranging from polar to nonpolar is crucial for optimizing extraction processes using three different extraction methods such as hydrodistillation, Soxhlet extraction, and UAE. This exploration allows for the selection of the most efficient solvent based on the polarity of the compounds of interest. The understanding how different solvents interact with plant materials aids in achieving higher yields and better extraction efficiency. It also provides valuable insights for various industries reliant on essential oils, such as pharmaceuticals and cosmetics. Fig. 2. Shows the percentage yields of essential oils extracted using three distinct methods, hydrodistillation, Soxhlet extraction, and ultrasonication-coupled with three different solvents (n-hexane, methanol, and ethyl acetate). In the traditional hydrodistillation method, which involves heating the plant material with water, n-hexane yielded

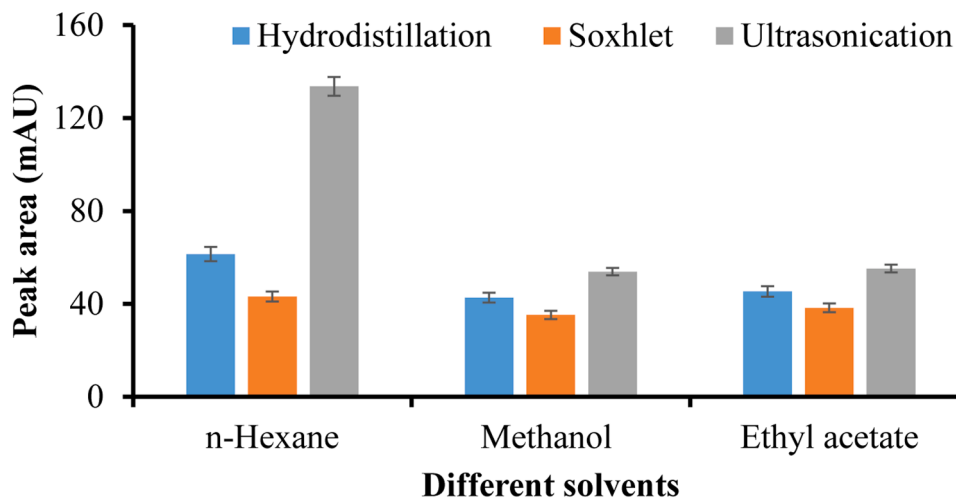


Fig. 3. Effect of different solvents and extraction methods for the extraction of β -caryophyllene from *Hyptis suaveolens*.

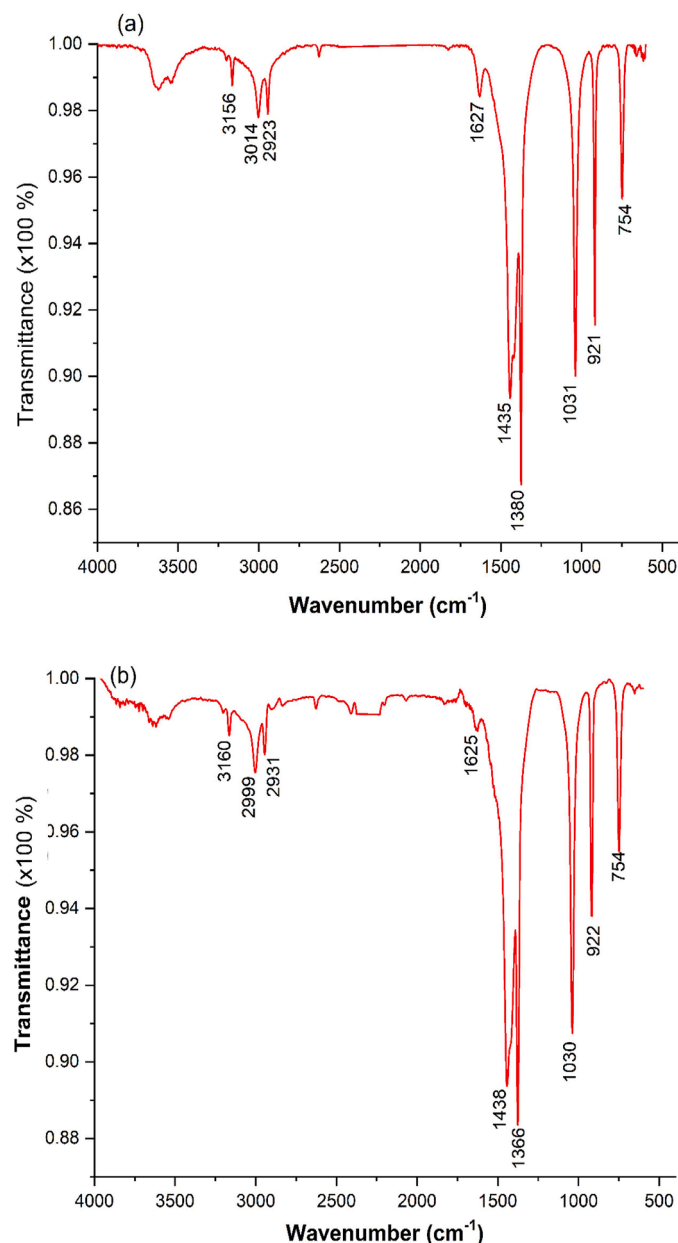


Fig. 4. FTIR spectrum of (a) standard β -caryophyllene; and (b) isolated β -caryophyllene using HPLC.

the highest percentage at 0.73 %, followed by ethyl acetate at 0.34 %, and methanol at 0.29 %. Soxhlet extraction, a continuous process using a solvent cycled through the sample, resulted in slightly lower yields, with n-hexane leading again at 0.46 %, followed by ethyl acetate at 0.31 %, and methanol at 0.25 %. Conversely, ultrasonication, employing ultrasonic waves to disrupt cell walls, produced significantly higher yields across all solvents: 8.08 % for n-hexane, 4.43 % for methanol, and 6.89 % for ethyl acetate. These results indicate that ultrasonication generally outperforms traditional extraction methods in terms of yield, with n-hexane consistently yielding the highest percentage of essential oils compared to methanol and ethyl acetate regardless of the extraction technique employed. n-Hexane is the optimal solvent for β -caryophyllene extraction due to its non-polar nature, which enhances solubility and prevents co-extraction of polar impurities. In contrast, methanol and ethyl acetate, being more polar, exhibit lower extraction efficiency and may extract unwanted compounds, reducing purity and yield in β -caryophyllene isolation [34,35]. Thus, n-hexane extracted

essential oil extracted through ultrasonication was employed for analysis of β -caryophyllene prior to HPLC analysis.

In the next study, the extraction efficiency of β -caryophyllene from essential oils was determined by the injecting the extracted essential oil into the HPLC. For this plant leaves of *Hyptis suaveolens* were selected for the determination of β -caryophyllene. The data from the HPLC analysis of peak areas mAU provides insights into the extraction efficiency of β -caryophyllene, a prominent compound in essential oils, using different solvents and extraction methods, shown in Fig. 3. For hydrodistillation, the highest extraction efficiency with peak area mAU of β -caryophyllene was 61.44, 42.67 and 45.33 when n-hexane, methanol and ethyl acetate were used as an extracting solvent, respectively. Similarly, Soxhlet extraction showed the highest efficiency with peak area mAU of 43.13, 35.22 and 38.29 for n-hexane, methanol and ethyl acetate, respectively. Notably, ultrasonication exhibited significantly higher extraction efficiencies across all solvents, with n-hexane, methanol and ethyl acetate for peak area of 133.61, 53.88 and 55.22, respectively. UAE with n-hexane as the most efficient method for β -caryophyllene extraction. UAE enhances yield by breaking plant cell walls through ultrasonic cavitation, reducing extraction time and solvent use. Steam distillation and Soxhlet extraction involve prolonged heating, risking compound degradation. Solvent polarity plays a crucial role, with n-hexane proving optimal [3,18,19].

3.3. Characterization of β -caryophyllene using FTIR analysis

The characterization of standard β -caryophyllene and isolated compounds through FTIR analysis is crucial for determining their structural composition and purity. FTIR spectroscopy provides valuable insights into functional groups present in unknown sample [23]. This ensures the quality and consistency of compounds used in various applications, including pharmaceuticals, cosmetics, and agrochemicals, enhancing their efficacy and safety profiles. The IR spectrum of isolated compound from *Hyptis suaveolens* was compared with the IR spectrum of pure β -caryophyllene standard in the range of 4000–400 cm^{-1} . The characteristic absorption peaks corresponding to stretching and bending vibrations of different functional group are shown in Fig. 4(A)4(B). FTIR bands in the range of 4000–1500 cm^{-1} showed functional group and various strong absorption bands in the range of 1500–500 cm^{-1} represented fingerprint region [36]. FTIR spectrum of β -caryophyllene exhibited absorption band of stretching vibrations at 3156 cm^{-1} of =CH. Similarly, absorption band at 1627 cm^{-1} represented stretching vibration of C = C bond. The spectrum showed paired band at 1435 and 1380 cm^{-1} represented symmetrical bending of $-\text{CH}_3$ group which can be attributed to two CH_3 group associated to the similar carbon atom. Methylene hydrogen form two $-\text{CH}$ stretching bands observed at 3014 cm^{-1} as an asymmetric stretching and 2923 cm^{-1} as a symmetric stretching which are specific for $-\text{CH}_2$ group. A strong band at 921 cm^{-1} indicated the out-of-plane deformation vibration of =CH [37]. After comparing the FTIR spectrum of pure β -caryophyllene standard and isolated compound by RP-HPLC analysis it was found that both spectra were resemblance and comparable to each other and the compound isolated from HPLC analysis was identified as β -caryophyllene.

3.4. Analytical evaluation for determination of β -caryophyllene using UAE- RP-HPLC

The method validation for β -caryophyllene determination in plant leaves using HPLC holds immense significance for ensuring reliable and accurate results. Establishing linearity across a broad concentration range enhances the method's applicability for various sample types and concentrations. Determining the LOD and LOQ ensures the method's sensitivity, allowing detection and quantification of β -caryophyllene at low concentrations. Precision assessments verify the method's repeatability and reproducibility, while accuracy evaluations validate the reliability of the results. This comprehensive validation process assures

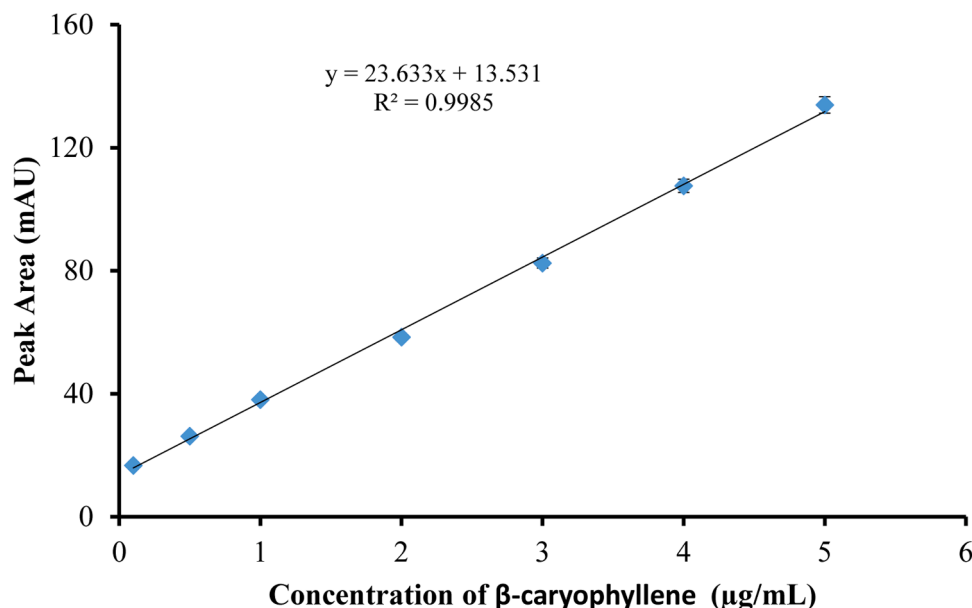


Fig. 5. Standard calibration curve of β-caryophyllene at optimized RP-HPLC conditions.

the quality of data obtained, enabling researchers to confidently quantify β-caryophyllene in plant leaves for pharmacological, ecological, and industrial purposes.

The method validation for quantifying β-caryophyllene in plant samples involved establishing a standard calibration curve. This curve was prepared using β-caryophyllene concentrations ranging from 0.1 to 5 μg/mL. The results demonstrated a linear relationship between β-caryophyllene concentration and peak area with a high correlation coefficient ($R^2 = 0.998$), indicating the method's linearity, specificity, and optimization for β-caryophyllene quantification, as depicted in Fig. 5. The limits of detection (LOD) and quantification (LOQ) were determined to be 0.02 and 0.07 μg/mL, respectively using the formula provided in experimental section. The precision assessment involved analyzing five replicates of β-caryophyllene standard in HPLC yielding a relative standard deviation of 0.45 %, indicating excellent precision. The accuracy of the method was evaluated by spiking a known concentration (1 and 2 μg/mL) of β-caryophyllene into pre-treated wild leaf basil (*Hyptis suaveolens*) samples, resulting in a recovery percentage of 98.5 to 102.1 %. These findings highlight the method's reliability and suitability for precise and accurate quantification of β-caryophyllene in complex plant matrices.

To assess potential interferences in the quantification of β-caryophyllene via HPLC, a variety of compounds (including α-pinene, β-pinene, methyl chavicol, 1,8 cineole, linalool, ocimene, borneol, geraniol, n-cinnamate, and eugenol) of concentration 10 mg/L spiked into solution containing followed by the HPLC analysis. There were no additional peaks appeared in the chromatogram at the retention times corresponding to the target analyte, it indicated the specificity of the method for measurement of β-caryophyllene in real samples. This interference study ensures the reliability and accuracy of the HPLC method by confirming its ability to selectively detect β-caryophyllene in complex sample matrices, free from interference from other substances.

3.5. Application of RP-HPLC assisted with UAE for determination of β-caryophyllene in different plant samples

The developed method was tested for quantification of β-caryophyllene in 14 plant samples. The extracted essential oil from different plant samples were diluted and injected for analysis of β-caryophyllene. The chromatographic peaks for β-caryophyllene in 14 plant samples are shown in Fig. S3. The concentration of β-caryophyllene

Table 1

Quantification of β-caryophyllene in selected plant samples using UAE and RP-HPLC-DAD method.

Name of plant samples	β-caryophyllene (g/100 g), (n = 3)	RSD, %
Clove (<i>Syzygium aromaticum</i>)	3.379±0.01	0.65
Coriander (<i>Coriandrum sativum</i>)	2.344±0.01	0.80
Cinnamon (<i>Cinnamomum verum</i>)	1.422±0.01	0.55
Curry leaves (<i>Murraya koenigii</i>)	3.587±0.01	0.66
Lemon (<i>Citrus lemon</i>)	3.965±1.20	0.43
Cumin (<i>Cuminum cyminum</i>)	3.329±1.00	0.70
Ajwain (<i>Trachyspermum ammi</i>)	3.642±1.11	0.55
Sweet basil (<i>Ocimum basilicum</i>)	1.252±1.01	0.62
Eucalyptus (<i>Eucalyptus citiodora</i>)	1.534±0.01	0.56
Holy basil (<i>Ocimum tenuiflorum</i>)	0.133±0.01	0.76
Indian bay leaf (<i>Cinnamomum tamala</i>)	2.691±0.01	0.65
Wild leaf basil (<i>Hyptis suaveolens</i>)	7.075±0.01	0.78
Rama Tulsi (<i>Ocimum sanctum</i>)	3.783±0.01	0.73
Satin wood (<i>Chloroxylon swietenia</i>)	0.053±0.01	0.62

present in different plant samples was estimated using standard calibration curve. Table 1 shows the concentration of β-caryophyllene in different plant samples. Each sample was analyzed in triplicate (n = 3), and the concentration of β-caryophyllene was reported in g/100 g. The results revealed varying concentrations of β-caryophyllene across the different plant samples. Wild leaf basil (*Hyptis suaveolens*) exhibited the highest concentration at 7.075±0.01 g/100 g, followed by Lemon (*Citrus lemon*) at 3.965±1.20 g/100 g and Rama tulsi (*Ocimum sanctum*) at 3.783±0.01 g/100 g. Conversely, Satin wood (*Chloroxylon swietenia*) displayed the lowest concentration at 0.053±0.01 g/100 g. This application demonstrates the effectiveness of RP-HPLC-DAD for accurate quantification of β-caryophyllene in various plant samples, providing valuable insights into their chemical composition and potential applications. The concentration of β-caryophyllenes was measured in the range of 0.053–7.07 g/100 g in the selected plant samples. Higher amount of β-caryophyllenes was found in *Hyptis suaveolens*.

3.6. Comparison of UAE-RP-HPLC with other reported method for determination of β-caryophyllene

The LOD, LOQ, linearity range, precision and accuracy obtained by the present method was evaluated to with other reported methods to

Table 2Comparison of UAE-RP-HPLC for determination of β -caryophyllene with other reported methods.

Extraction methods	Analytical techniques	Linearity range ($\mu\text{g mL}^{-1}$)	LOD ($\mu\text{g mL}^{-1}$)	LOQ ($\mu\text{g mL}^{-1}$)	RSD, %	Recovery, %	Samples	Ref.
Steam distillation	HPLC-DAD	0.250–20.0	0.125	0.250	–	100.6 %	Clove	[39]
Steam distillation	GC	5.04 – 201.6	1.28	3.89	0.95–1.31	101.6 – 102.2	Clove	[28]
–	GC-FID	0.1 – 8.9 (mM)	2.8	–	4.9	100	Conifer derived essential oil	[41]
Hydro - distillation	RP-HPLC-PDA	10–100	2.5	10	1.658	100.0	Ageratum conyzoides	[40]
Solid liquid extraction	GC-MS	0.625–40	–	–	–	–	Melissa officinalis L.	[42]
–	HPLC-DAD	64–96	0.789	2.629	0.79–1.44	98.4	Cordia verbenacea	[26]
Traditional method	HPLC-UV	5–11	0.119	0.398	1.34	99.1– 100.5	Copaiba oleoresin	[27]
Ultra-sonication extraction	RP-HPLC-DAD	0.1 – 5	0.016	0.055	0.45	102.1	14 plant samples	Present work

know the potentiality of the newly developed method (Table 2). Notably, the developed method exhibits superior performance in key parameters such as LOD, LOQ and precision. When compared to reported methods concerning *Cordia verbanacea* and *Copaiba oleoresin*, the values of LOD, LOQ, RSD %, and recovery % align closely with those of the present method [26,27]. Furthermore, methods utilizing HPLC-DAD and HPLC-PDA for estimation of β -caryophyllene in clove and *Ageratum conyzoides* demonstrate comparatively higher value of LOD and LOQ than the present study [38,39]. GC and GC-FID have also been employed for quantification of β -caryophyllene though less sensitive for detection of target analyte compared to the present study [28,40,41]. Thus, comprehensive comparison highlights the superiority of the developed RP-HPLC-DAD method in delivering more accurate analyses of β -caryophyllene in essential oils extracted from selected plant samples.

3.7. Application of β -caryophyllene in insecticidal activity by determination of mortality rate (%) against stored grains pest

β -caryophyllene, a natural sesquiterpene found in many essential oils such as clove, rosemary, and black pepper, exhibits significant insecticidal properties. Its application in insect control has been widely explored, with studies demonstrating its efficacy against various pests including mosquitoes, ants, and agricultural pests. β -caryophyllene acts through multiple mechanisms, including disruption of insect neurotransmitter systems and interference with insect growth and development. Its natural origin and low toxicity to mammals make it an attractive alternative to synthetic pesticides, offering potential for environmentally friendly pest management strategies. Additionally, its repellent properties make it useful in formulations aimed at preventing insect infestations in both agricultural and residential settings [42–44]. Further research into its modes of action and formulation techniques could lead to the development of novel insecticides with enhanced efficacy and safety profiles.

The insecticidal activity of β -caryophyllene against stored grains pests, as evidenced by the provided data (Table S1), showcases its potential as an effective means of pest control. The experiments conducted on both *Sitophilus granarius* and *Corcyra cephalonica* larvae reveal a clear trend of increasing mortality rates with higher concentrations of β -caryophyllene and longer exposure periods. For *Sitophilus granarius*, mortality rates escalate from 0 % at 1 h to as high as 95 % at 48 h with a 500 mgL^{-1} concentration. Similarly, *Corcyra cephalonica* larvae exhibit increasing mortality rates, reaching 98 % at 48 h with the same concentration. This trend underscores the efficacy of β -caryophyllene as an insecticide, demonstrating its ability to significantly reduce pest populations within relatively short time frames. These findings suggest that β -caryophyllene holds promise as a natural and potent solution for combating stored grains pests, offering potential benefits for agricultural practices seeking safer and more sustainable pest management strategies [33,45,46].

4. Conclusions

A simple, sensitive and rapid UAE method was developed for quantitative determination of β -caryophyllene from essential oils of 14 plant samples using RP-HPLC. The UAE method showing higher extraction efficiency of essential oils compared to traditional methods, such as steam distillation or hydrodistillation. Additionally, the RP-HPLC-DAD method developed in this study proved significant for the separation and identification of β -caryophyllene in 14 selected plant samples, showing better LOD LOQ values compared to previously reported methods, along with a good linearity range. This improved method can thus be used for the quantification of β -caryophyllene in various plant samples such as roots, seeds, flowers, and fruits. Furthermore, β -caryophyllene was found to be an effective insecticide against two stored grain pests, *Sitophilus granarius* and *Corcyra cephalonica* larvae. Therefore, our study suggests that β -caryophyllene can be utilized as an effective biopesticide for managing these pests.

The RP-HPLC-DAD method demonstrates excellent performance in separating and quantifying β -caryophyllene, offering high sensitivity, precision, and reproducibility. However, its stability and long-term performance across various practical applications require further validation to ensure consistent results. Additionally, exploring the method's applicability for analyzing other sesquiterpenes and structurally similar volatile compounds would broaden its utility in essential oil analysis, pharmaceutical research, and natural product studies. Future studies should focus on optimizing conditions for different plant matrices and expanding its application to diverse bioactive compounds.

CRediT authorship contribution statement

Reena Jamunkar: Writing – original draft, Methodology, Formal analysis. **Deepak Sinha:** Writing – review & editing, Supervision. **Kamlesh Shrivastava:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Tarun Kumar Patle:** Writing – review & editing. **Antresh Kumar:** Writing – review & editing. **Khushali Tandey:** Writing – review & editing. **Tribhuvan Singh:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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Data availability

provided as supplementary material

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