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Essential oil yield and antioxidant activity in diseased and healthy betel leaves: A comparative study of bioactive compounds using GCMS and FTIR profiling

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Abstract

This study focuses on the extraction of essential oils from healthy and diseased betel leaves (*Piper betle* L.), revealing novel insights into the potential value of diseased leaves, which are often discarded. The findings demonstrate that diseased betel leaves retain a substantial amount of essential oil content, challenging the conventional practice of disregarding them in favour of healthy leaves. Using hydrodistillation, the oil yield from healthy leaves was 1.52%, whereas the yield from diseased leaves was 1.23%. Despite this reduced yield, the chemical composition of the oils revealed unique stress responses in diseased leaves. GC-MS analysis revealed that healthy Betel Leaf Essential Oil (BLEO) contained higher concentrations of key compounds, including copaene (40.91%) and chavibetol (31.85%). At the same time, diseased BLEO contained elevated levels of stress-related compounds such as gamma-muurolene and eucalyptol. This increase in gamma-muurolene and eucalyptol suggests that the diseased leaves undergo biochemical changes in response to stress, adding significant value to their oil content. FTIR analysis confirmed these compositional differences, with diseased BLEO showing reduced peak intensities for critical aromatic and phenolic compounds. Antioxidant activity assays (DPPH and ABTS) indicated that while healthy BLEO exhibited slightly better antioxidant properties (IC50 values: 4.04 µL/mL and 3.41 µL/mL), diseased BLEO retained considerable antioxidant potential (IC50 values: 4.79 µL/mL and 3.73 µL/mL). This research highlights the importance of reconsidering diseased betel leaves as a valuable source of essential oil, offering opportunities for sustainable resource utilization and increased economic benefits by utilizing generally discarded leaves.

Key words: Antioxidant activity, betel leaf essential oil, GC-MS analysis, FTIR analysis

Introduction

The betel leaf (*Piper betle* L.), a perennial climber belonging to the Piperaceae family, holds significant cultural and medicinal importance, especially in Asia (Roy and Guha, 2021). The use of betel leaves dates back thousands of years, playing an integral role in traditional practices and rituals. Its leaves are commonly chewed with areca nut and slaked lime in many Asian cultures, a practice believed to enhance social interaction and stimulate digestion (Gunjal *et al.,* 2020). However, beyond its cultural applications, the betel leaf is known for its medicinal properties, attributed mainly to its essential oil content (Guha and Nandi, 2019). Betel leaf essential oil is a rich source of bioactive compounds that contribute to its wide range of therapeutic properties (Vikhas *et al.,* 2012). The major components identified in betel leaf essential oil include eugenol, acetyl eugenol and caryophyllene, which exhibit various biological activities (Madhumita *et al.,* 2019). Eugenol, for instance, is well-known for its analgesic, anti-inflammatory, and antimicrobial properties (Nisar *et al.,* 2021). Chavibetol has been documented for its antifungal and antibacterial effects, while caryophyllene is recognized for its potent antioxidant activity. Antioxidant activity is one of the crucial therapeutic properties of betel leaf essential oil. Antioxidants are compounds that inhibit oxidation, a chemical reaction that can produce free radicals, leading to cell damage (Sen *et al.,* 2010). The presence of antioxidants in betel leaf essential oil helps in neutralizing these free radicals, thereby reducing oxidative stress and preventing various chronic diseases such as cancer, cardiovascular diseases, and neurodegenerative disorders (Roy and Guha, 2021; Das *et al.,* 2022; Sanubol *et al.,* 2016; Pradhan *et al.,* 2013). The essential oil extracted from betel leaves serves medicinal purposes and holds economic value. The industrial production of essential oils has significant applications in pharmaceuticals, cosmetics, and aromatherapy sectors (Singh *et al.,* 2023). With its unique blend of bioactive compounds, betel leaf essential oil is particularly valued for its potential in developing natural health products and preservatives.

Maintaining the health of betel plants is crucial for optimizing both the yield and quality of essential oil (Nantitanon *et al.*, 2007). However, diseases such as leaf spot, stem rot, and anthracnose pose significant challenges reducing both the quantity and quality of leaves (Kumar *et al.*, 2016; Maiti and Sen, 1979). While most farmers discard disease-affected leaves, unaware of their potential oil content and quality, this practice leads to economic loss and the underutilization of valuable resources. Notably, there is a gap in the literature regarding how diseases alter the composition and bioactivity of betel leaf essential oil, particularly the antioxidant properties and oil yield. This study addresses that gap by comparing healthy and diseased betel leaf oil composition and antioxidant activity. This is essential since farmers discard nearly 10-20% of their yield as diseased leaves in every harvest. By exploring how plant health

influences essential oil properties, we aim to answer critical questions: How do diseases affect the bioactive compounds in betel leaves, and can these oils still hold medicinal and economic value? This research is highly relevant to regions where betel leaf cultivation is a major agricultural activity. Sustainable farming practices, such as utilizing diseased leaves that are usually discarded, can help reduce waste and improve economic outcomes for farmers. By maximizing the use of healthy and diseased leaves, this study enhances the economic value of betel cultivation and promotes resource efficiency (Sarkar and Solanki, 2017). Furthermore, understanding the impact of plant diseases on oil composition may support breeding programs for disease-resistant betel varieties, ensuring sustainable and resilient production. Betel leaf essential oil, known for its significant medicinal and economic potential (Mondal, 2022; Sahu *et al.*, 2022; Peddapalli *et al.*, 2020; Pandey *et al.*, 2023), can significantly benefit from such comprehensive research. This study will provide insights into optimizing the utilisation practices by comparing the biochemical properties of oils from healthy and diseased leaves, thus unlocking the full potential of this valuable resource for producers and consumers alike.

Materials and methods

Collection of betel leaves: Healthy and disease-affected betel leaves were collected from a betel vine germplasm conservatory (called in local vernacular as "Pana Baraja") established in Fakir Mohan University, Balasore new campus (21.544° N, 86.8159° E). Betel leaves were collected using a stratified random sampling method to ensure representative samples from both healthy and diseased categories. The diseased leaves were affected explicitly by leaf spot disease (a fungal disease), identified by visual symptoms such as discolouration, necrotic spots, and irregular growth patterns. Healthy leaves showed no visible signs of infection or stress. A total of 300 leaves were collected, divided equally between healthy (n = 150) and diseased (n = 150) samples. The sample size of 150 leaves (for each category) was chosen to provide a robust comparison, ensuring statistical reliability and representativeness and to account for potential variability in oil content across leaf conditions. This sampling strategy minimized bias and maximized the comparability of results between the healthy and diseased groups. After collection, the leaves were shade-dried, a critical step since drying time has been shown to impact essential oil yield, with slower drying preserving higher oil content. This ensured that any variation in oil content between healthy and diseased leaves was due to the condition of the leaves and not external drying factors. After that, the leaves were finely powdered using a mechanical grinder to facilitate extraction.

Extraction of essential oil: A total of 100 g of the betel leaf powder was subjected to hydrodistillation to obtain the essential oil by using Clevenger apparatus. The powdered leaves were placed in a 2L distillation flask and distilled water in a ratio 1:10 w/v appropriate for hydrodistillation. A heating mantle heated the mixture, and the volatile oil compounds were carried over with steam into the condenser. The distillation continued until no more essential oil was collected. The distillate, comprising essential oil and water, was collected in a receiver. The essential oil, being immiscible with water, was separated by decantation. The essential oil was dried by anhydrous sodium sulphate and stored in a refrigerator at 4 °C for further analysis. The betel leaf essential oil yield was calculated by the following equation 1.

$$\text{Essential oil yield (\%)} = \frac{\text{The weight of extracted oil(g)}}{\text{Weight of betel leaf taken in experiment(g)}} \times 100 \quad (1)$$

GC-MS analysis: The essential oil samples extracted from betel leaf were analysed using gas chromatography-mass spectrometry (SHIMADZUGCMS-QP2020NX). This method separates the volatile oil compounds via gas chromatography (GC) and identifies them at a molecular level using the mass spectrometer (MS) component. To prepare for the analysis, 20 µL of the essential oil was dissolved in 980 µL of methanol. From this solution, 1 µL was injected into the GC/MS system to identify the chemical compounds present. The experimental conditions for the GC/MS analysis were as follows: the column dimensions were 30 m × 0.25 mm with a film thickness of 0.25 µm. Helium was used as the carrier gas at a 1 mL/min flow rate. The oven temperature program began with an initial injection temperature of 60°C, held for 1 min, followed by an increase of 4°C per minute until reaching 220°C. The temperature was then maintained at 220°C for 10 minutes, threshold 70 eV with the mass range from 50-600 m/z, resulting in a total run time of 35 min. The injection temperature was set at 230°C, while the MS transfer line was kept at 280°C. Samples (1 µL) were injected into the GC column in splitless mode. The identification of chemical compounds was verified by comparing the obtained mass spectra with the NIST 11 library (Madhumita *et al.*, 2019).

Statistical analysis: A one-way ANOVA was conducted using Minitab 17 software to assess the data, followed by a Tukey HSD (Honestly Significant Difference) test. This analysis revealed differences in the percentage of bioactive components present in both healthy and diseased betel leaves. Data within each row that had distinct superscript letters were considered significantly different at a significance level of $P < 0.05$

FTIR analysis: All samples were kept at room temperature (25°C) for 30 min before the FTIR analyses (SHIMADZU IRSpirit-X). A single reflection ATR accessory was used for all spectral acquisitions. The spectral measurement parameters were set with a resolution of 4 cm^{-1} and an accumulation of 50 scans. Instrument control and data acquisition were managed using Lab solution IR program. Each sample was placed on a diamond ATR crystal using a pipette. The ATR crystal was cleaned with methanol before each spectral acquisition, and the background air spectrum was scanned prior to each acquisition.

Antioxidant Activity

DPPH: The DPPH assay for the essential oil was conducted by the method of Swapna *et al.* (2012). A 0.1 mM DPPH solution in methanol was prepared, and 1.0 mL of this solution was mixed with 1.0 mL of the essential oil solution in methanol, with concentrations ranging from 2 to 10 µL /mL, as well as with commercial antioxidants (ascorbic acid). The reaction mixture was thoroughly vortexed, then left in the dark at 25°C for 30 min before measuring the absorbance at 517 nm by using UV-VIS spectroscopy (SHIMADZU UV2600i). The ability of the essential oil to scavenge the DPPH radical was calculated as percentage inhibition using the following equation (Madhumita *et al.,* 2019).

$$\text{DPPH free radical scavenging activity (I\%)} = \frac{A_c - A_t}{A_c} \times 100$$

Where A_c and A_t represent the test and control samples, respectively.

The scavenging activity versus essential oil sample concentration was demonstrated on a graph, where the essential oil concentration providing 50% inhibition (IC_{50}) was determined.

ABTS method: In the ABTS free radical assay, the method of Re *et al.* (1999) was adopted with minor modifications, specifically diluting the ABTS stock solution in methanol. The pre-formed radical cation of ABTS was generated by oxidizing a 7 mM ABTS solution with a 2.45 mM potassium persulfate solution in equal volume. The mixture was allowed to react for 16 h in the dark at 25°C. Then 1mL of the resulting solution was diluted in 60 mL of methanol to achieve an absorbance of 0.706 ± 0.001 at 734 nm. Then 1 mL of the ABTS radical cation solution was added to betel leaf essential oils solutions at concentrations of 2, 4, 6, 8, 10 µL/mL, as well as to commercial antioxidants (ascorbic acid) prepared in methanol, and the absorbance was measured at 734 nm. The oil's percentage inhibition of the ABTS radical was calculated using the equation described in the DPPH assay. The scavenging activity versus essential oil sample concentration was demonstrated on a graph, where the essential oil concentration providing 50% inhibition (IC_{50}) was determined.

Results and discussion

Yield of essential oil: The essential oil yield from betel leaves varies with leaf health. Using hydrodistillation with a Clevenger apparatus, healthy betel leaves produce 1.527 ± 0.030 g of essential oil per 100 g of dry leaves (1.52%), while diseased leaves yield only 1.236 ± 0.039 g (1.23%) (Fig. 1). This reduction can be attributed to physiological and biochemical disruptions caused by infection. Pathogenic attacks compromise photosynthetic efficiency and biomass production, leading to altered metabolic pathways. Resources are diverted from secondary metabolite synthesis, including essential oils, to defence mechanisms. Pathogens can induce reactive oxygen species (ROS), causing oxidative stress and damaging oil-producing glands.

Additionally, infections impair nutrient and water uptake, further stressing the plant. Consequently, the energy allocated for essential oil synthesis is redirected to producing pathogenesis-related proteins and other defensive compounds. This downregulation of the biochemical machinery responsible for essential oil biosynthesis results in a significant decrease in yield from diseased betel leaves compared to healthy ones.

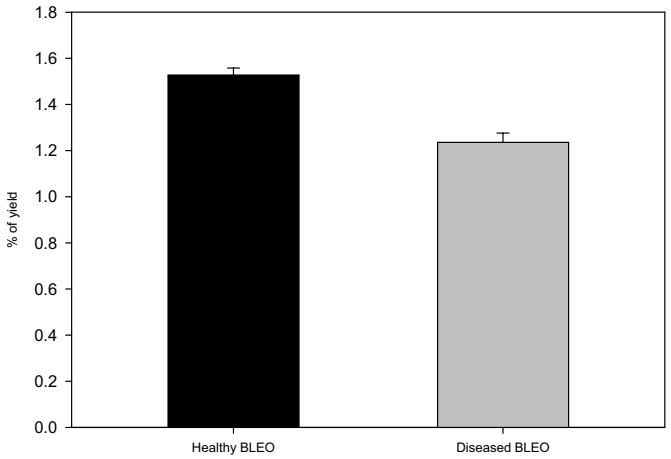

Fig. 1. Essential oil yield from healthy and diseased betel leaves

GC-MS analysis: The Gas Chromatography-Mass Spectrometry (GC-MS) analysis of bioactive compounds in healthy and diseased Betel Leaf Essential Oil (BLEO) revealed notable shifts in their chemical profiles, shedding light on how disease alters the biosynthetic pathways of the plant (Fig. 2a, b). In healthy BLEO, the major compounds included Copane (41.53%) and Chavibetol (31.80%). However, in diseased BLEO, these compounds decreased to 33.39% and 28.04%, respectively. Simultaneously, several compounds significantly increased in concentration, suggesting a stress-induced metabolic response (Table 1). For instance, gamma-muurolene increased by more than two times, rising from 5.51% to 11.45%, and eucalyptol increases from 0.37% to 1.29%. These increases could serve as potential biomarkers for plant stress response, offering novel insights into how the plant copes with disease. Other compounds, such as D-Limonene, Linalool, and Bourbonene, also show an upward trend in diseased BLEO, while new compounds like Eugenol Acetate appeared in the healthy state at 1.5%. The rise of gamma-muurolene and Eucalyptol, combined with the reduction of key compounds like Copaene and Chavibetol, highlights the disease's impact on BLEO's chemical composition and could suggest a disruption in essential oil synthesis pathways.This chemical profile shift has broader commercial implications. The enhanced levels of certain bioactive compounds in diseased BLEO could offer new economic opportunities. For example, gamma-muurolene and Eucalyptol are known for their therapeutic properties, and their elevated presence in diseased leaves might increase the market value of BLEO under stress conditions. This could help mitigate economic losses typically associated with diseased plants. Furthermore, recognizing and leveraging these changes could lead to more sustainable cultivation practices, where even diseased leaves hold commercial value. Understanding these disease-induced variations is crucial for developing strategies to optimize the quality of BLEO, ensuring its continued therapeutic and economic potential, even under adverse conditions.

FTIR analysis: The FTIR analysis of healthy and diseased Betel Leaf Essential Oil (BLEO) provides essential insights into their chemical compositions. The FTIR spectra for both types confirmed the presence of eugenol, chavibetol, and linalool, as established by reference to the FTIR library (Fig. 3a, b). Characteristic absorption bands identified these compounds. For example, the broad absorption around 3600-3200 cm^{-1} corresponds to O-H stretching vibrations, indicating the presence of alcohols and phenols in both oils. Peaks around 2929-2854 cm^{-1} represent aliphatic C-H stretching vibrations, suggesting a similar hydrocarbon framework in both healthy and diseased BLEO. Significant differences occured in the 1600-1500 cm^{-1} region, associated with C=C stretching vibrations typical of aromatic compounds like eugenol and chavibetol (Dhoot *et al.*, 2009). The healthy BLEO showed more pronounced peaks here, implying higher concentrations or a more intact structure of these aromatic compounds. In the C-O stretching region (1260-1050 cm^{-1}), the healthy BLEO exhibited stronger absorption bands, indicating a higher or more stable content of ethers and phenolic compounds.GC-MS analysis supports the FTIR findings by

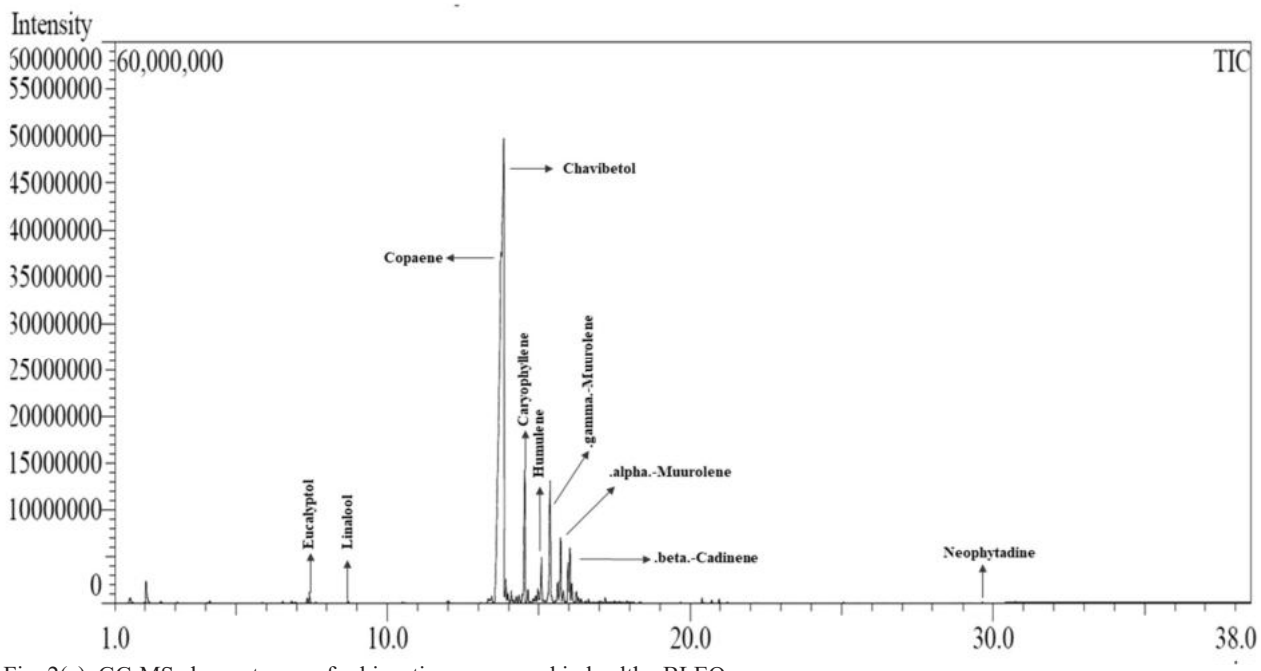

Fig. 2(a). GC-MS chromatogram for bioactive compound in healthy BLEO

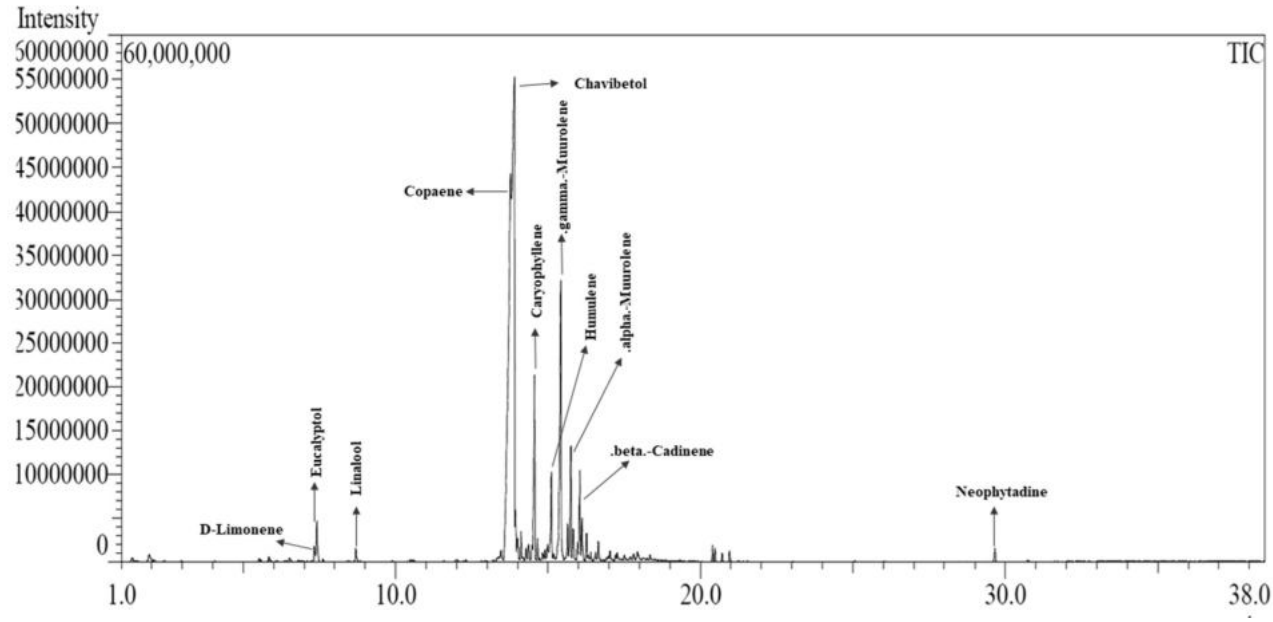

Fig. 2(b). GC-MS chromatogram for bioactive compound in diseased BLEO

identifying copaene, caryophyllene, humulene, and gamma-muurolene. These sesquiterpenes are characterized by absorption bands in the C-H stretching (3000-2850 cm^{-1}) and fingerprint regions (1500-500 cm^{-1}), further validated by the FTIR spectra (Dekermenjian, 1999). Peaks at 1450-1375 cm^{-1} indicate C-H bending vibrations, confirming these hydrocarbons in healthy and diseased oils. Overall, while both healthy and diseased BLEO contain similar compounds, the diseased BLEO typically exhibits lower intensity peaks, suggesting reduced concentrations or degradation of certain compounds, which may be due to disease. This comprehensive analysis highlights the impact of plant health on the essential oil's chemical profile, crucial for understanding its therapeutic and commercial value.

Antioxidant activity: The antioxidant activity of healthy and diseased BLEO was assessed using the DPPH and ABTS radical scavenging assays, with IC_{50} values determined for both samples. The IC_{50} value indicates the concentration required to scavenge 50% of the free radicals present, with lower values reflecting higher antioxidant activity. In the DPPH assay, the IC_{50} for healthy BLEO was 4.04 µL/mL (Fig. 4a), while diseased BLEO had a slightly higher IC_{50} of 4.79 µL/mL (Fig. 4b) suggesting that healthy BLEO has marginally better antioxidant capacity. Similarly, in the ABTS assay, the IC_{50} values were 3.41 µL/mL for healthy BLEO (Fig. 5a) and 3.73 µL/mL for diseased BLEO (Fig. 4b). Both assays indicated that healthy BLEO outperformed diseased BLEO, albeit by a small margin. The control IC_{50} value for ascorbic acid, a standard antioxidant, was 8.42 µL/mL by the DPPH method. Both healthy and diseased BLEO showed significantly lower IC_{50} values compared to ascorbic acid, demonstrating strong antioxidant properties regardless of the health status of the leaves. The higher IC_{50} values in diseased BLEO may be linked to the reduced concentration of chavibetol, a known antioxidant (Khuntia *et al.*, 2023). Chavibetol content

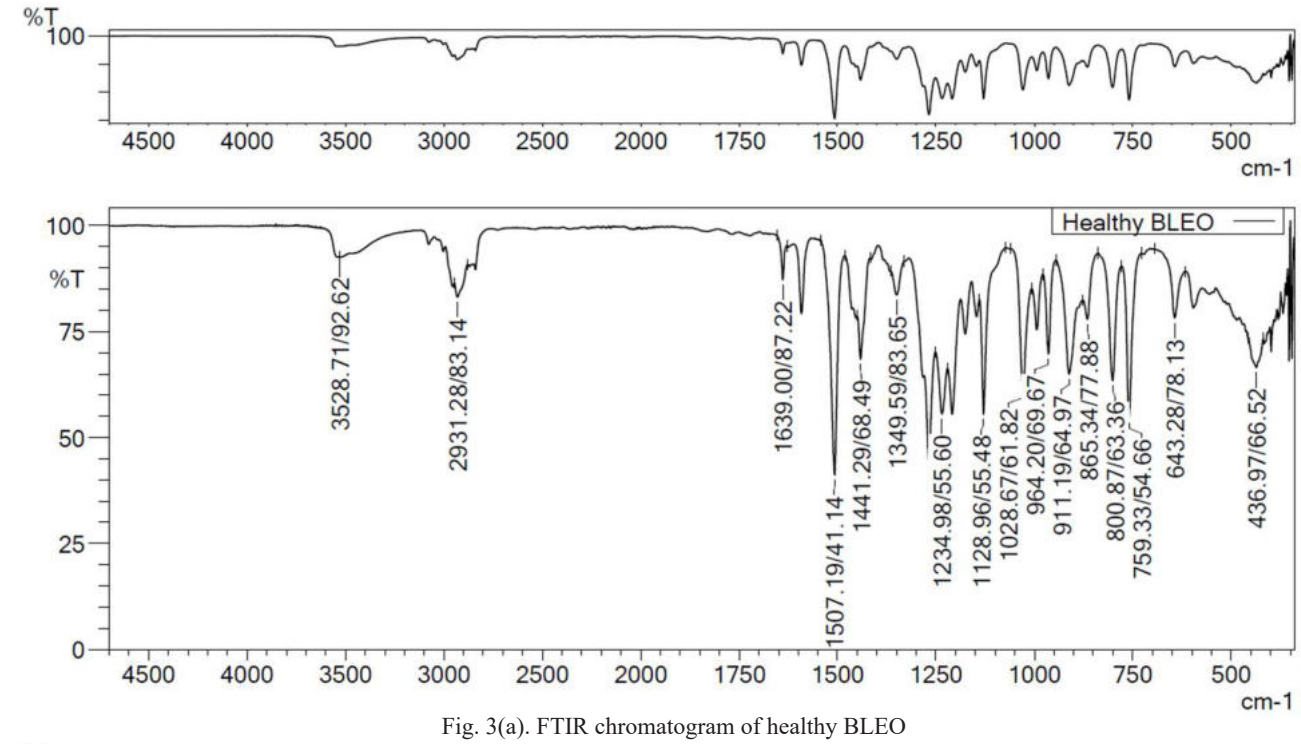

Fig. 3(a). FTIR chromatogram of healthy BLEO

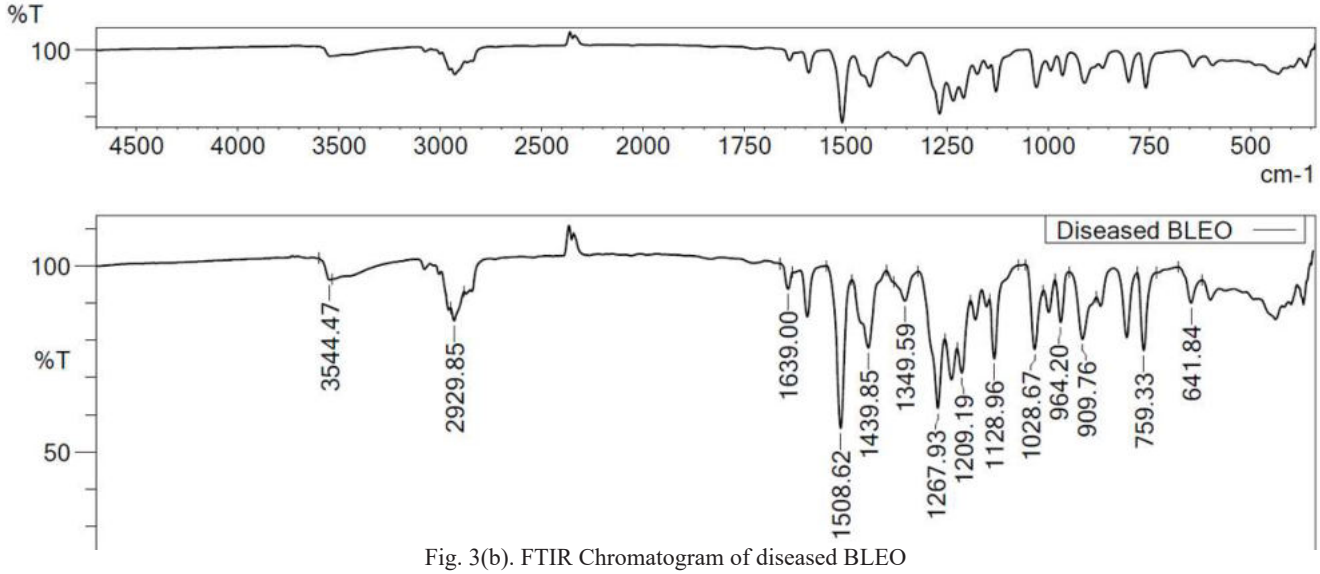

Fig. 3(b). FTIR Chromatogram of diseased BLEO

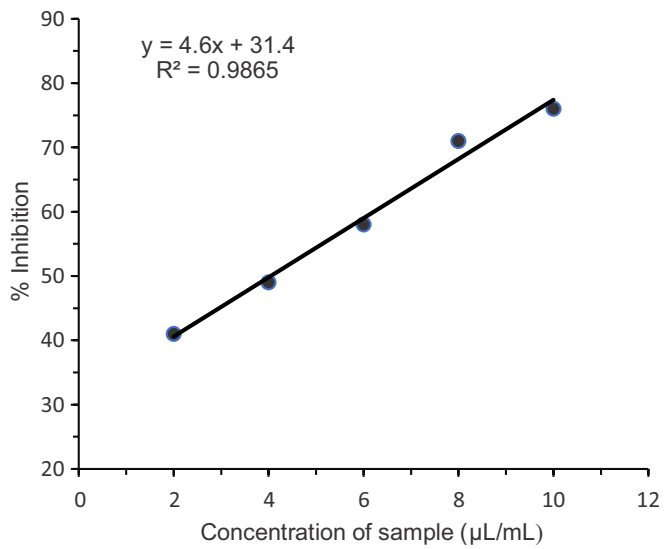

Fig. 4(a). Antioxidant activity of healthy BLEO by DPPH method

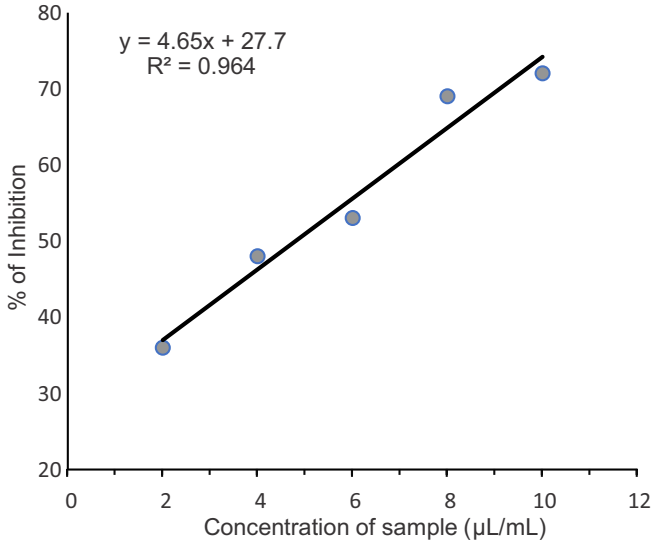

Fig. 4(b). Antioxidant activity of diseased BLEO by DPPH method

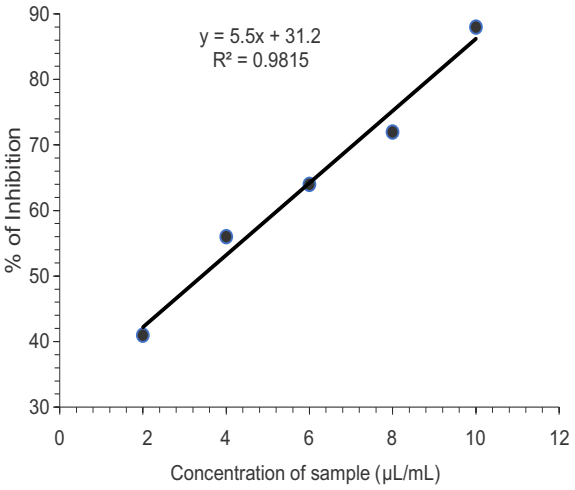

Fig. 5(a). Antioxidant activity of healthy BLEO by ABTS method

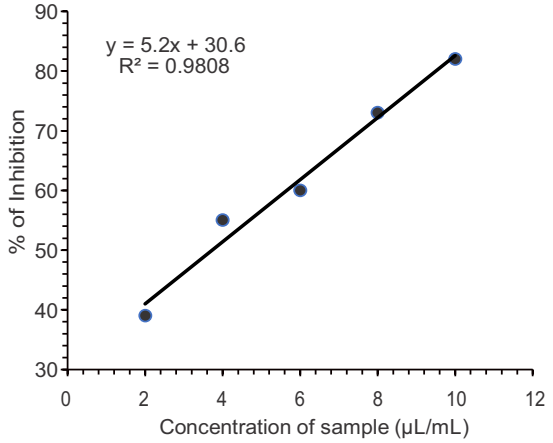

Fig. 5(b). Antioxidant activity of diseased BLEO by ABTS

Table 1. Major bioactive compounds (with % of area) in healthy and diseased BLEO

Sl. No.	Compound name	RT (min)	Healthy BLEO % of area	Diseased BLEO% of area	Nature of the compound
1	D-Limonene	7.3	0.18±0.01 $^{i-k}$	0.49±0.03 $^{f-h}$	Monoterpene
2	Eucalyptol	7.4	0.37±0.02 $^{h-k}$	1.29±0.06 ^{f}	Monoterpenoids
3	Linalool	8.6	0.08±0.01 ^{k}	0.38±0.03 $^{f-h}$	Monoterpenoids
4	Copaene	13.7	41.53±0.63 ^{a}	33.39±0.83 ^{a}	Sesquiterpenes
5	Chavibetol	13.8	31.80±0.76 ^{b}	28.04±1.14 ^{b}	Phenylpropanoids
6	Beta.-Bourbonene	13.9	0.89±0.07 ^{gh}	1.08±0.02 $^{f-h}$	Sesquiterpenes
7	Elemene	13.97	0.36±0.02 $^{h-k}$	0.73±0.31 $^{f-h}$	Sesquiterpenes
8	Methyl Eugenol	14.0	0.42±0.03 $^{h-k}$	0.54±0.05 $^{f-h}$	Phenylpropanoids
9	Bergamotene	14.45	0.36±0.03 $^{h-k}$	0.51±0.02 $^{f-h}$	Sesquiterpenes
10	Caryophyllene	14.53	4.77±0.07 ^{d}	5.25±0.06 ^{d}	Terpenes
11	Santalene	14.63	0.48±0.05 $^{h-k}$	0.44±0.05 $^{f-h}$	Sesquiterpenes
12	Aromandendrene	14.81	0.75±0.04 $^{h-j}$	0.89±0.03 $^{f-h}$	Monoterpenes
13	Farnesene	14.89	0.24±0.02 $^{i-k}$	0.42±0.04 $^{f-h}$	Sesquiterpenes
14	Humulene	15.08	1.87±0.06 ^{ef}	2.69±0.05 ^{e}	Sesquiterpenes
15	γ-Muurolene	15.37	5.51±0.16 ^{c}	11.45±0.26 ^{c}	Sesquiterpenes
16	γ-gurjunene	15.54	0.8±0.06 ^{hi}	1.14±0.09 $^{f-h}$	Sesquiterpenes
17	α -muurolene	15.72	2.41±0.04 ^{e}	2.88±0.07 ^{e}	Sesquiterpenes
18	β -Bisabolene	15.80	0.55±0.03 $^{h-k}$	1.00±0.02 $^{f-h}$	Sesquiterpenes
19	Eugenol Acetate	15.96	1.5±0.06 ^{fg}	0	Phenylpropanoid
20	β -Cadinene	16.02	1.77±0.07 ^{ef}	2.83±0.09 ^{e}	Terpenes
21	Calamenene	16.08	0.82±0.03 ^{hi}	1.22±0.11 ^{fg}	Sesquiterpenes
22	Cubenene	16.24	0.40±0.03 $^{h-k}$	0.73±0.03 $^{f-h}$	Sesquiterpenes
23	α -calacorene	16.39	0.19±0.02 $^{i-k}$	0.24±0.01 ^{h}	Sesquiterpenes
24	Caryophyllene oxide	16.64	0.11±0.01 ^{jk}	0.69±0.02 $^{f-h}$	Sesquiterpenes
25	Neophytadine	20.38	0.32±0.02 $^{h-k}$	0.34±0.03 ^{gh}	Diterpenes

Data in the column with distinct superscript letters are significantly different using one way ANOVA followed by Turkey HSD test ($p \leq 0.05$). Values are represented in the form of Mean ± SD. RT=Retention time

was 31.85% in healthy BLEO but decreased to 27.37% in diseased BLEO, resulting slight decline in antioxidant capacity. As a phenolic compound, chavibetol is vital for scavenging free radicals and enhancing BLEO's overall antioxidant activity (Gupta and Guaha, 2023; Swapna *et al.*, 2012).

This study presents a novel comparison between healthy and diseased betel leaves for essential oil extraction, addressing a critical gap in understanding how disease affects both oil yield and bioactivity. The findings reveal that healthy leaves produce slightly higher oil content, but essential oil extracted from diseased leaves retains a substantial amount of key bioactive compounds such as eugenol, chavibetol, linalool, copaene, caryophyllene, humulene, and gamma-muurolene, confirmed through comprehensive GC-MS and FTIR analyses. Despite a marginal reduction in antioxidant capacity, as measured by DPPH and ABTS assays, the IC_{50} values of diseased leaf oil remain close to those of healthy leaves, indicating significant antioxidant activity. The study highlights the potential for sustainable agricultural practices by demonstrating that diseased betel leaves, generally discarded, can still contribute economic value. This underexplored resource could be utilized in essential oil production, offering new avenues for generating revenue from what is usually considered as waste. Such findings are particularly relevant for regions dependent on betel leaf cultivation, suggesting that diseased leaves should be viewed as a valuable resource rather than agricultural waste, enhancing the sustainability and profitability of betel farming.

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Authors' contribution: Conceptualization and designing of the research work (SKD, SKG); Execution of experiments and data collection (SKD, SKG), Analysis of data (SKG). All authors contributed to the article and approved the submitted version.

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