

## COMPARATIVE METABOLOME PROFILING OF POPULAR BETEL LEAF (*PIPER BETLE L.*) LANDRACES OF WEST BENGAL

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### ABSTRACT

Betel-leaf creeper is a prominent horticultural cash crop of West Bengal with a sizeable contribution towards the agro-economy of the state. Farmers propagate several elite cultivars of betel-leaf for extraction of high quality essential oil. The study focused on the comparative analysis of popular landraces on account of essential oil content (volume/fresh-weight) and its putative metabolome profile. The 'Sagar Meetha' genotype (0.24%) produced maximum oil followed by 'Kakdwip Meetha' (0.21%), 'Kali Bangla' (0.18%) and 'Sada Bangla' (0.14%) respectively from fresh leaves. The GC-MS/FID analysis of the leaf oil revealed thirty-four compounds with chavicol, chavibetol, methyl chavicol and  $\beta$ -caryophyllene as the major volatiles. The methyl chavicol (estragole) fraction was higher in 'Meetha' types than 'Bangla' producing the sweet fragrance. The absence of  $\beta$ -Bourbonene in Sagar Meetha, presence of (Z)- $\beta$ -ocimene (0.1%) and leaf alcohol (0.1%) in Kalibangla ecotypes were identified as major discriminants. The eugenol content was low in coastal cultivars. The PCA identified two Principal components for regulating 80% of the variation of cultivars and HCA analysis confirmed genetic fidelity of the selected landraces. The information could be useful for the suitable utilization of the betel-leaf landraces for extraction of known and novel chemical constituents in pharmaceutical and cosmetic industries.

**Keywords:** Betel-leaf landraces, essential oil content, GC-MS/FID, metabolome profile, PCA-HCA analysis, industrial utilization

### INTRODUCTION

*Piper betle* L. is grown profusely in different parts of West Bengal and is regarded as one of the most important contributor to agro-forestry system of the state. Betel leaf is a prominent cash crop earning equal importance in fresh trade and pharmaceutical sector. The leaves of different cultivars of betel-leaf produces signature fragrance due to production of a mixture of numerous volatile and non-volatile chemical components in multiple combinations within the oil glands and the constituents have significant value in cosmetic industries as the potent marker for natural aroma (Pise *et al.*, 2022). This heart-shaped leaf is propagated for 15–20 million Indian and 2 billion foreign consumers annually. The crop provides Rs 6000–7000 million of national income per year and at the same time supplies leaves worth Rs 30–40 million in global export (Das *et al.*, 2016).

The leaves are not only used for chewing purposes but the leaf essential oil is used in medicine, stimulant, antiseptic, tonic and other ayurvedic formulations (Mondal, 2022a). The betel leaf essential oil (BLEO) is used from ancient times in food-sector (Tran *et al.*, 2020), medicine (Roy and Guha, 2018) and perfume industry (Preethy *et al.*, 2017). The metabolome profiling of the popular landraces could identify potential contributor landraces for natural extraction of numerous bioactive compound valuable for diverse industries. West Bengal is one of the notable states producing huge amount of betel leaf with alarming post-harvest loss amounting up to 70% of the total produce including domestic and export market (Guha, 2006). The perishability and fluctuation in the fresh betel leaf market affects the betel trade influencing the cultivation of this important crop. The utilization of excess leaves for extraction of leaf essential oil could reduce the post-harvest loss and boost the betel farmers to uplift the rural economy.

The previous studies on qualitative profile of essential oil of betel creeper has revealed the presence of phenyl-propanoid class of chemicals with strong anti-bacterial (Nayaka *et al.*, 2021), anti-fungal (Makkar *et al.*, 2017), anti-inflammatory (Alfiana *et al.*, 2022), antioxidant (Das *et al.*, 2022), anti-protozoan (Sarkar *et al.*, 2008), anti-cancer (Paranjpe *et al.*, 2013), aphrodisiac (Fazal *et al.*, 2014), immune-stimulant (Saputra *et al.*, 2023), larvicidal (Setiawan *et al.*, 2019) and insecticidal properties (Gupta and Guha, 2024). The betel-leaf cultivars store

a number of valuable volatile components related to the characteristic fragrance and pharmaceutical properties of the plant. The components include eugenol, chavicol, linalool, estragole,  $\alpha$ -copaene, caryophyllene, anethole, eucalyptol,  $\alpha$ -muurolene, valencene, caryophyllene oxides,  $\gamma$ -cadinene, cubebene, isoeugenol acetate, methyl eugenol,  $\alpha$ -phellendrene,  $\beta$ -elemene,  $\beta$ -cardinene, chavicol (Suryasnata *et al.*, 2016; Madhumita *et al.*, 2019; Islam *et al.*, 2020). Multiple factor affects the deposition of different secondary metabolites in leaf glands of the plant (Karak *et al.*, 2016). In this paper four popular betel leaf landraces were collected from different zones of South 24 Parganas district of West Bengal to assess the chemical constituents of the extracted essential oil for further utilization of the landraces in medicinal, food and cosmetic industries.

### MATERIALS AND METHODS

#### Plant material and extraction of essential oil

In South 24 Parganas region four betel-vines (baroj) were marked for the present experiment (Tab 1). Fresh betel leaves of four prominent genotypes 'Sagar Meetha', 'Kakdwip Meetha', 'Kali Bangla', and 'Sada Bangla' were collected from Sagar island, Kakdwip, Zulpia and Amtala region of South 24 Parganas district of West Bengal, India and brought to the Genetics & Plant Breeding laboratory of School of Agriculture & Allied Sciences, The Neotia University for further study and analysis. The betel creepers were marked inside the vines (baroj) and herbarium of the leaf specimens were preserved in the departmental laboratory. The identification of the cultivars was done according to the DUS characteristics available for betel-leaf germplasm (Alam *et al.*, 2023). Leaves were collected randomly from 20 creepers from each betel-vine, mixed and utilized for extraction of essential oil in a Clevenger-type apparatus using the modified method of hydro-distillation (Mondal, 2022b). The extraction was done with five replications for each cultivar for finalizing the essential oil content.

**Table 1** Geographic description of the selected betel-vines for genotype collection

Cultivar Name with symbol	Location	Village	Farmer	Latitude	Longitude	Altitude (M)	Cultivation Type
Kali Bangla (B1)	Zulpia, West Bengal	Fingadhowri	Pranay Samanta	21.8760° N	88.1853° E	4	Closed baroj
Sada Bangla (B2)	Amtala, West Bengal	Kripampur	Sajal Sardar	22.3669° N	88.2777° E	9	Closed baroj
Sagar Meetha (M2)	Sagar Island, West Bengal	Chakphuldupi	Baladeb Mandal	21.7269° N	88.1096° E	4	Closed baroj
Kakdwip Meetha (M1)	Kakdwip, West Bengal	Srinagar	Matin Sheikh	21.8760° N	88.1853° E	4	Closed baroj

**Analysis and characterization of the essential oil constituents**

The leaf essential oil extracted from different cultivars of betel leaves were utilized for chemical analysis. The component analysis of the essential oil was done using GC-MS & FID techniques. The analysis of the leaf essential oil was performed in CMAP, Lucknow. Gas Chromatography was carried out on Perkin Elmer Autosystem XL Gas Chromatograph 8500 series equipped with Flame ionization detector (FID) and a head space analyser using a fused silica capillary RTX-5 column (30 m X 0.32 mm, film thickness 0.25 µm) with dimethyl polysiloxane RT<sub>X5</sub>. Oven temperature was programmed from 60 to 280°C with injector temperature 230°C and detector temperature 250°C. Injection volume 1µl, hydrogen was used as carrier gas (1.0ml/min). The identification of individual compounds was carried out using retention indices (RI) derived from gas chromatograms by comparing with a homologous series of n-alkanes (C<sub>7</sub> to C<sub>30</sub>, Supelco Bellefonte, PA, USA). Compounds were identified with literature values by computer matching against library spectra built using pure substances and components of known essential oil and final validation was done by matching the mass spectra with those of recorded in MS library (NIST Chemistry Web Book) and literature with interpretation of the constituent profile of betel-leaf landraces (Adams et al., 1995). The principal component analysis (PCA) and the Hierarchical cluster analysis (HCA) was performed with Minitab, 2021 (www.minitab.com) for tree diagram generation and heat map analysis of chemical compounds were performed with NCSS11 statistical software (2016; NCSS, LLC.; ncss.com/software/ncss).

**RESULTS AND DISCUSSION**

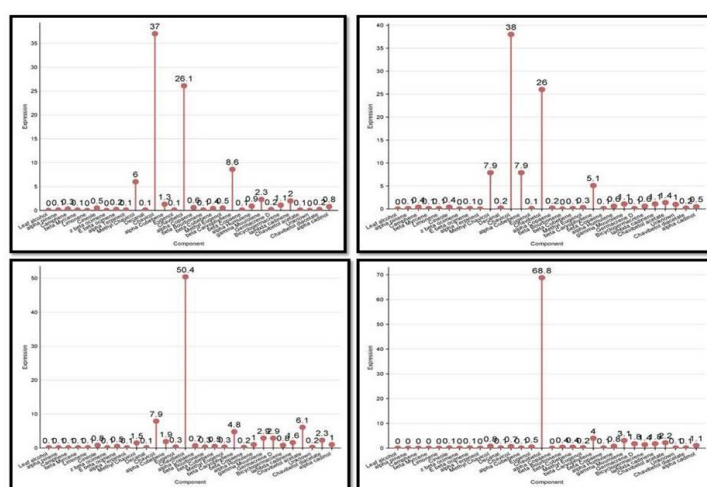
**Leaf Oil Essential Content**

The extraction of leaf essential oil was done with time duration of 3 hours with a leaf: solvent (distilled water) ratio of 1:3 with a fixed temperature of 80±1°C. Our study showed significant variation in the total leaf essential oil (LEO) content among the four prominent landraces of West Bengal. Maximum amount of essential oil (v/w) was recovered from ‘Sagar Meetha’ (0.24%) genotype followed by ‘Kakdwip Meetha’, ‘Kali Bangla’ and ‘Sada Bangla’ with yield of 0.21%, 0.18% and 0.14% respectively from fresh leaf samples. The cultivars included in this study are profusely grown in the South 24 Parganas district of West Bengal. The estimation of total betel leaf essential oil content is important for initiating post-harvest processing unit of betel leaves with its downstream utilization. The district with saline coastal belt is known for its distinct betel-leaf landraces with ample prospect for creation of roadmap for betel-leaf essential oil based aroma business.

**Chemometric analysis of Leaf Essential Oil**

The Gas Chromatography and Mass Spectroscopy analysis of the extracted essential oil revealed the presence of thirty-four compounds in leaf essential oil

(Tab 2). The unknown components were identified using the NIST library data. The major fraction of the essential oil exhibited the presence of chavicol, chavibetol, methyl chavicol and β-caryophyllene in the most widely grown cultivars. The GC-FID analysis was used for exact quantification of the identified carbon compounds. The chavicol fraction was higher in ‘Meetha’ genotypes (Kakdwip Meetha 37% and Sagar Meetha 38%) than ‘Bangla’ types. The ‘Bangla’ ecotypes showed highest amount of chavibetol (Kali Bangla 50.4%, Sada Bangla 68.8%) followed by chavicol and β-caryophyllene (Figure 1).



**Clockwise from leaf side: Kakdwip Meetha (M1), Sagar Meetha (M2), Upper row Kalibangla (B1), Sada Bangla (B2) Lower row**

**Figure 1** The metabolome profile of Leaf Essential Oil of the four betel-leaf landraces analysed through GC-MS/FID analysis

In this study the chemical profile of *Ocimum basilicum* was used as the reference metabolome for comparison of the genotypes. The *Ocimum* genotype is known for production of phenyl propanoid class of volatiles. The present analysis proclaimed that chavicol and chavibetol could act as the important biomarker for the popular betel-leaf cultivars ‘Meetha’ and ‘Bangla’ of West Bengal. The chavicol, chavibetol ratio may act as an indicator in differentiation of ‘Meetha’ and ‘Bangla’ landraces. The percentage of methyl chavicol (estragole) is high in ‘Meetha’ landraces than ‘Bangla’ type. The estragole fraction relates to the characteristic sweet fragrance of ‘Meetha’ landraces of both Sagar Island and Kakdwip region. Previous studies also revealed either estragole or anethole defines the characteristic sweet fragrance of ‘Meetha’ betel-leaf (Guha and Nandi, 2019).

**Table 2** Essential Oil Composition of the selected betel leaf landraces

SL No.	Constituent	RI <sup>a</sup>	RI <sup>b</sup>	M1	M2	B1	B2	IM
1	Leaf alcohol	851	851	T	T	0.1	T	RI,MS
2	α-pinene	933	932	0.1	0.1	0.1	T	RI,MS
3	Camphene	949	946	0.3	0.4	0.1	T	RI,MS
4	Sabinene	973	969	T	T	T	T	RI,MS
5	β-Myrcene	990	988	0.1	0.1	0.1	T	RI,MS
6	p-Cymene	1024	1020	T	T	T	T	RI,MS
7	Limonene	1029	1024	T	0.1	0.1	T	RI,MS
8	1,8-Cineole	1031	1026	0.5	0.4	0.8	0.1	RI,MS
9	(Z)-β-ocimene	1035	1032	T	T	0.1	T	RI,MS
10	(E)-β-ocimene	1046	1044	0.2	0.1	0.5	0.1	RI,MS
11	γ-Terpinene	1057	1054	T	T	T	T	RI,MS

12	Terpinolene	1088	1086	T	T	T	T	RI,MS
13	Linalool	1099	1095	0.8	0.1	0.6	0.5	RI,MS
14	Camphor	1141	1144	Nd	Nd	Nd	Nd	RI,MS
15	Terpinen-4-ol	1177	1174	T	T	T	T	RI,MS
16	$\alpha$ -Terpineol	1191	1186	0.1	T	0.1	T	RI,MS
17	Methyl Chavicol	1200	1195	6.0	7.9	1.5	0.8	RI,MS
18	Decanal	1204	1201	0.1	0.2	0.1	0.1	RI,MS
19	Chavicol	1256	1247	37.0	38.0	7.9	0.7	RI,MS
20	Safrole	1291	1285	T	T	T	T	RI,MS
21	$\alpha$ -Cubebene	1347	1345	1.3	7.9	1.9	0.1	RI,MS
22	Eugenol	1358	1356	0.1	0.1	0.3	0.5	RI,MS
23	Chavibetol	1376	1370	26.1	26.0	50.4	68.8	RI,MS
24	$\alpha$ -Copaene	1377	1374	0.6	0.2	0.7	T	RI,MS
25	$\beta$ -Bourbonene	1385	1387	0.1	T	0.3	0.4	RI,MS
26	$\beta$ -Cubebene	1391	1387	0.4	0.1	0.5	0.4	RI,MS
27	Methyl Eugenol	1405	1403	0.5	0.3	0.3	0.2	RI,MS
28	$\beta$ -Caryophyllene	1421	1417	8.6	5.1	4.8	4.0	RI,MS
29	$\beta$ -Copaene	1433	1430	0.1	0.1	0.2	0.1	RI,MS
30	$\alpha$ -Humulene	1453	1452	0.9	0.6	1.0	0.8	RI,MS
31	$\gamma$ -Muuroleone	1477	1478	2.3	1.1	2.9	3.1	RI,MS
32	Germacrene D	1481	1484	0.2	0.1	2.9	1.8	RI,MS
33	Bicylogermacrene	1495	1500	1.1	0.6	0.8	1.4	RI,MS
34	$\delta$ -cadinene	1523	1522	2.0	1.1	1.6	1.8	RI,MS
35	Chavibetol acetate	1527	1524	0.1	1.4	6.1	2.2	RI,MS
36	Unknown	1557	-	T	1.0	0.2	0.1	RI,MS
37	Chavibetol diacetate	1644	-	0.2	0.2	2.3	0.1	RI,MS
38	$\alpha$ -cadinol	1654	1652	0.8	0.5	1.0	1.1	RI,MS
39	Unknown	1744	-	T	T	T	T	RI,MS
				90.4	94.4	90.3	89.3	RI,MS

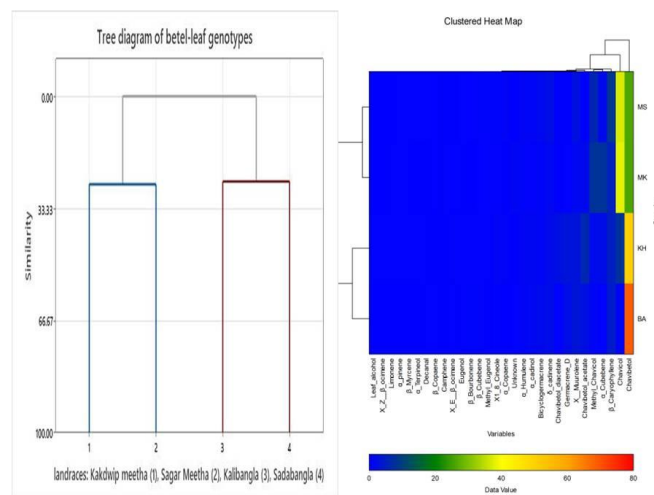
RP: calculated Retention Index in Elite-5 capillary columns using a homologous series of n-alkanes (C7-C30 hydrocarbons) and RP: Retention Index in published library (R. P. Adams, 1995) t; trace  $\leq 0.1\%$  IM; identification method based on the GC-FID results (RI) and mass-spectrometry detection (MS) t: trace; nd: not detected; Suvaas Data was taken from the published work "Chemical composition of phenylpropanoid rich chemotypes of *Ocimum basilicum* L. and their antimicrobial activities". <https://doi.org/10.1016/j.indcrop.2022.114978>.

The investigation highlighted the absence of  $\beta$ -Bourbonene in 'Sagar Meetha' and presence of (Z)- $\beta$ -ocimene (0.1%) and leaf alcohol (0.1%) in 'Kalibangla' landrace. The individual leaf essential oil profile was sufficient to analyze the value of the selected cultivars in aroma-business. The cultivars of West Bengal were devoid of Safrole, Sabinene,  $\gamma$ -Terpinene and Terpinolene prominently found in South Indian and Sri Lankan genotypes (Mondal, 2022e). The percentage of eugenol and germacrene D are also less compared to cultivars of Orissa and South Indian varieties validating the ecotypic divergence of the chemotypes owing to environmental influence and clonal propagation.

### Principal component Analysis and Hierarchical Cluster Analysis with Constituent Heat Map generation

In order to better, understand the variability of the chemical constituents the Principal component analysis (PCA) was performed with the leaf essential oil. The scree plot, score plot, loading plot and biplot generated by Minitab ver. 2021, stated that the first principal component (PC1) explained 48.7% and second principal component (PC2) covered 31.7% of the total variation of extracted oil. In the PCA analysis, the first two components explained about 80% of the total variation and the compounds that played significant role are chavibetol, eugenol,  $\beta$ -bourbonene,  $\beta$ -cubebene,  $\alpha$ -humulene and  $\gamma$ -muuroleone among the 31 components identified through GC-MS/FID analysis (Figure 2).

The four betel-leaf landraces were widely grown in different parts of West Bengal mainly in South 24 Parganas. The hierarchical cluster analysis (HCA) was performed with the same dataset using Minitab software for the selected cultivars for understanding the chemotypic fidelity. The dendrogram was constructed with Euclidean distance with complete linkage and standardized variables. The four genotypes separated into two distinct groups, one cluster covering Kakdwip and Sagar Meetha cultivars and the other cluster with Kalibangla and Sadabangla. The tree diagram confirmed the accurate clustering of the chemotypes with respect to the leaf oil profile. The field and morphological variation exhibited by the landraces were in accordance with the chemical distinction revealed in our study. The heat map generated with NCS statistical software using the chemical components also provides a quick visual representation of the concentration of different metabolites present in leaf essential oil with variable colour intensity along with clustering information of the selected genotypes with respect to the major and minor chemicals extracted through hydro-distillation.



**Figure 2** The tree diagram of landraces based on similarity index and chemotypic variability displayed by heat map analysis of the selected landraces of betel-leaf

In a study done by Gochhi and his co-worker with six popular land races of Orissa including 'Haldi', 'Birkuli', 'Chandrakana', 'Aima', 'Nuagaon' and 'Dhinkia' showed the presence of highest amount of eugenol (Gochhi and Dey, 2024). It was noticed that the essential oil of the selected Orissa cultivars were rich in phenyl-propanoid compounds with eugenol, gamma-muuroleone and caryophyllene as the main constituents. The highest amount of oil was extracted from 'Haldi' while lowest from 'Birkuli' landrace. The study enumerated the industrial prospect of diverse cultivars of betel-leaf in food and pharmaceutical industries. The maximum eugenol content was found in 'Chandrakana' succeeded by 'Aima' and 'Haldiran'. Gamma-muuroleone and caryophyllene recovery was high in 'Dhinkia', 'Nuagaon' and 'Aima'. In the present study, the recovery of eugenol was low in the South Bengal landraces confirming the difference between landraces of Orissa and West Bengal.

The genotypes of Bangladesh revealed presence of eugenol and beta-caryophyllene as the main constituent of the betel leaf essential oil. Five varieties

yielded 101 compounds with 42 common constituent noticed in all varieties. The research claimed the pioneering report of detection of 50 volatile compounds from leaves of betel varieties of Bangladesh. Oxophorone and 9-epi-beta-caryophyllene detected in 'Bangla' genotype of Bangladesh that was not detected in the 'Bangla' genotypes of West Bengal, India. The Principle component analysis from the studied chemical profile of essential oil grouped 'Misti' and 'Bari Pan 3' in the same cluster, 'Sanchi' and 'Khasia' in another cluster along with 'Bangla' type. The research exhibited the proximity of 'Bangla', 'Sanchi', 'Khasia' and secluded them from 'Misti' and 'Bari Pan 3' (Islam et al., 2020). Our study also grouped the 'Meetha' and 'Bangla' cultivars in diverse clusters based on leaf essential oil profile.






In a study conducted by Karak et al. (2016) involving eight betel leaf cultivars of Purba Medinipur district of West Bengal identified the presence of a total of 75 constituents. The study included both polar and non-polar components with application of Principle component analysis and Partial-Least-Square-Discriminants analysis. Eugenol, eugenyl acetate, β-caryophyllene, δ-elemene, γ-murolene, α-humulene, germacrene D, bicylogermacrene, α-cubebene and β-copaene were present in all ecotypes with fluctuating relative response ratio with respect to season. Their study showed the presence of chavicol and estragol in 'Meetha' cultivar. The 'Meetha' and 'Chhaanchi' cultivar formed a single cluster separating from the rest including 'Bangla', 'Bagerhati', 'Manikdanga', 'Kalibangla', 'Ghanegete' and 'Haldi' in another group. In our study, the presence of estragole fraction was higher in 'Meetha' types than the 'Bangla' leaves confirming the compound as volatile marker for the distinct sweet fragrance qualifying as an olfactory marker in some specific landraces.

In a recent study on sex discrimination of *Piper betle* L. landraces highlighted identification of some compounds such as phenol and organic acid with prevalence

in male creepers whereas gallic acid, sinapic acid, caffeic acid, tartaric acid, citric acid, and malonic acid as common phytochemical identified as gender-neutral components. Twenty genotypes screened for determination of compounds effective as sex index. The research identified prospective genotypes for commercial cultivation along with volatile signature related to gender of this dioecious creeper (Narayanappa et al., 2024). The present study also identified Kalibangla as a special chemotype with leaf alcohol, (Z)-β-ocimene and α-Terpineol.

In our study, five components were detected as major contributor in the leaf essential oil profile of selected genotypes. The oil is predominated by phenyl propanoid class of compounds along with lesser quantities of sesquiterpenes and monoterpenes. The phenyl propanoids are effective anti-pathogenic compound aiding wound healing, shielding of uv-rays and inflammation having six-carbon aromatic phenyl group and a three-carbon propene tail (Korkina, 2007). The Germacrene are sesquiterpenes were well known for their anti-cancer properties (Abu-Izneid et al., 2020; Di Sotto et al., 2020). The betel-leaves propagated for fresh and raw consumption as well as used in diverse industries. The complete volatile signature of the leaf essential oil provided information regarding its usage in pharmaceutical sector and prospect of the creeper in substitution of some important medicinal plants such as anise, nutmeg, galangal, cinnamon, clarkia, tulsi, tarragon, oregano, gardenia in nutraceutical extraction (Tab 3). This study could assist the farmers in selection of suitable climate resilient cultivars for propagation for aroma-industries with lucrative volatile signature. Additionally, this research effort for the first time presented the complete account of secondary metabolite profile of the leaf essential oil of elite genotypes of the district of South 24 Parganas of state of West Bengal of India.

**Table 3** Details of the major components present in betel leaf essential oil

Name	Chemical Nature	Chemical Structure	Molecular Weight (g/mol)	Alternate Plant source	Uses	Reference
Chavicol	Phenyl propene		134.01	Anise, gardenia	Antioxidant, anticancer	Oliveira et al., 2021; Panneerselvam et al. 2022
Chavibetol	Phenyl propanoid		164.20	Galangal, Clarkia	Flavor, weedicide, antioxidant	Kemprai et al., 2023
Methyl Chavicol	Phenyl propene		148.20	Basil, anise, tarragon	Anti-lipase, anti-oxidant, anti-depressant	Dwivedi and Tripathi 2014, Benitez et al., 2009
β-caryophyllene	Bicyclic sesquiterpenes		204.35	Oregano, cinnamon	Anti-cancer, anti-oxidant, anti-bacterial, neuro-protective	Dahham et al. 2015, Machado et al. 2018
Methyl Eugenol	Phenyl propene		178.23	Nutmeg, mace	Flavoring agent, antibacterial	Goswami et al. 2017

**CONCLUSION**

The metabolome profile of the essential oil from four betel-leaf landraces of West Bengal gave the complete information sufficient to assess their applicability in pharmaceutical and cosmetic industries. The 'Bangla' and 'Meetha' types exhibited difference in major volatile signature. The state of West Bengal produces huge amount of betel leaves but faces 70-75% post-harvest loss of the cash crop. The utilization of the surplus leaves for extraction of essential oil and simultaneous utilization of the same in industrial value addition processes could contribute one more natural product in wellness medicine sector. Additionally, this research work for the first time provided an insight on essential oil content and chemotypic signature of popular landraces of coastal saline belt of West Bengal.

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