International Journal of Agricultural Technology 2026 Vol. 22(2):817-830
DOI: https://doi.org/10.63369/ijat.2026.22.2.817-830
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Influence of leaf type, genotype, and location on bioactivities and assessment of genetic diversity in teak (*Tectona grandis* L. f.)

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Poeaim, S. and Phonmakham, J. (2026). Influence of leaf type, genotype, and location on bioactivities and assessment of genetic diversity in teak (*Tectona grandis* L. f.). International Journal of Agricultural Technology 22(2):817-830.

Abstract Marked variation in biological activities was observed among teak (*Tectona grandis* L. f.) leaf extracts as influenced by leaf type, tree genotype, and planting location. Fresh leaves generally exhibited stronger antibacterial activity than fallen leaves, with significant differences detected among plus trees originating from Chiang Mai, Phrae, Sukhothai, Lampang, and Khon Kaen and grown at the Thongphaphum and Phitsanulok Silviculture Research Stations. Among all samples, the Phrae plus tree cultivated at Thongphaphum showed the highest antibacterial inhibition against the tested microorganisms. Methanol extracts demonstrated antibacterial, anti-tyrosinase, and anti-inflammatory activities, while sequential extraction of Phrae plus tree leaves using hexane and dichloromethane yielded fractions with potent antibacterial effects, indicating promising potential for the development of new antibacterial agents. Genetic analysis based on sequence-related amplified polymorphism (SRAP) markers revealed similarity coefficients ranging from 0.67 to 1.00, reflecting low to moderate genetic diversity among the studied germplasm, with some samples exhibiting nearly identical genetic profiles. Overall, the observed variation in biological activities was closely associated with both genetic background and environmental conditions, providing valuable information for teak germplasm selection and the utilization of leaf-derived bioactive compounds in product development.

Keywords: Antibacterial activity, Bioactivity, Genetic diversity, SRAP markers, Teak

Introduction

Plants and medicinal herbs have long been used to treat diseases and promote health. Despite the widespread availability of synthetic chemicals and antibiotics, herbal products remain popular because they are perceived as safe and provide multiple health benefits. Plant-derived extracts can be developed into pharmaceuticals, nutraceuticals, and cosmetics. Consequently, modern research focuses on isolating bioactive compounds, characterizing their chemical and physical properties, and evaluating pharmacological activity to support the development of value-added products.

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Teak (*Tectona grandis* L. f.), a deciduous species in the family Verbenaceae, is native to Southeast and South Asia, including Thailand, Myanmar, Laos, Indonesia, and Southern India. The part of the teak tree—bark, wood, flowers, fruits, and leaves—has been reported to possess medicinal properties. Previous studies have documented a range of biological activities of teak extracts, including antibacterial (Purushotham and Sankar, 2013; Lanka and Parimala, 2017; Phonmakham *et al*., 2018), antioxidant (Mosad *et al*., 2014), anti-inflammatory and analgesic (Nayeem and Karvekar, 2009), and hypoglycemic effects (Varma and Jaybhaye, 2010). Methanolic leaf extracts contain phenolic compounds such as gallic and ellagic acids and flavonoids, including rutin and quercetin, all known for their antioxidant and antibacterial activities (Nayeem and Karvekar, 2010).

However, plant extracts' biological activity varies with genetic and environmental factors such as genotype, planting location, and developmental stage. For example, phenolic content and antioxidant capacity in Olive leaves change with harvest month (Brahmi *et al*., 2015), Moringa (*Moringa oleifera*) leaves from different regions of Pakistan display distinct antioxidant profiles (Iqbal and Bhenger, 2006), and young Aronia leaves contain higher polyphenol and flavonoid levels than mature leaves (Thi and Hwang, 2014). These examples illustrate that genetic background and growing conditions can markedly influence the bioactive potential of plant materials.

Molecular markers are powerful tools for exploring such genetic variation. In India, studies using random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) markers revealed broad genetic diversity among teak provenances, with similarity coefficients ranging from 0.31 to 0.85 for RAPD and 0.27 to 0.88 for ISSR, and little correspondence between genetic clustering and geographic origin (Narayanan *et al*., 2007). Altitude has also been linked to population structure: RAPD analysis of six Indian sites separated populations into two altitude-related clusters (Murukan and Murugan, 2015). Sequence-related amplified polymorphism (SRAP) markers detected similarity coefficients between 0.11 and 0.92 among 32 accessions from Gujarat and grouped them into seven clusters at a 0.49 similarity (Thakor *et al*., 2019).

Comparable work in Thailand shows moderate genetic differentiation. RAPD analysis of teak populations in Thailand revealed considerable genetic diversity among provenances, highlighting substantial intraspecific variation (Changtragoon and Szmidt, 2000). Pongkrawee and Volkaert (2012) assessed the genetic diversity and population structure of 25 natural teak populations in Thailand using eight single-strand conformation polymorphism (SSCP) markers. The analysis revealed a high level of genetic variation (79% polymorphic loci; expected heterozygosity = 0.39) and identified four distinct genetic groups,

providing essential information for conserving and managing teak genetic resources in Thailand. These studies confirm that molecular markers are valuable for assessing teak genetic variation, although the extent and geographic pattern of diversity differ among regions. Microsatellite analysis of 29 natural teak provenances across its native range revealed a substantial eastward decline in genetic diversity from India toward Southeast Asia and a well-defined population structure, supporting India as the species'center of origin (Fst = 0.227; G″st = 0.632) (Hansen *et al*., 2015). Among available molecular tools, the SRAP technique developed by Li and Quiros (2001) is beneficial for assessing plant genetic diversity. Using specially designed forward and reverse primers to amplify open reading frame regions of genomic DNA preferentially, SRAP provides rapid and reproducible detection of multiple polymorphic loci without prior sequence information.

The present study investigated how leaf type (fresh versus fallen leaves), plus-tree genotype, and planting location influence teak leaf extracts' antibacterial, anti-tyrosinase, and anti-inflammatory activities. The genetic diversity of selected teak plus trees was evaluated using SRAP markers to clarify their genetic structure.

Materials and methods

Plant material for bioactive analysis, extraction and fractionation

Fresh and fallen teak leaves from five plus trees located in Chiang Mai, Phrae, Lampang, Sukhothai, and Khon Kaen were collected from two planting sites in Thailand: the Thongphaphum and Phitsanulok Silviculture Research Station. Among these, crude methanol extracts of leaves from the Phrae plus tree displayed the most potent antibacterial activity (Phonmakham *et al*., 2018) and were selected for further fractionation by liquid–liquid extraction to obtain more purified extracts. Four samples of Phrae plus tree leaves—fresh and fallen from both Thongphaphum and Phitsanulok stations (PTF, PTD, PPF, and PPD)—were sequentially extracted with five solvents: hexane (H), dichloromethane (D), ethyl acetate (E), n-butanol (B), and water (W), yielding 20 solvent fractions. Bioactivity screening revealed that the hexane and dichloromethane fractions from the Thongphaphum station exhibited the highest activities. These active fractions were subsequently subjected to silica gel column chromatography. The mobile phase system was optimized based on thin-layer chromatography (TLC), and eluates were monitored under UV light (254 and 366 nm) and visualized with anisaldehyde reagent. Fractions with similar TLC profiles were pooled and designated as sub-fractions. Extract codes comprise four characters: the first

represents the solvent (H, D, E, B, or W), the second the plus tree (P), the third the planting location (T for Thongphaphum, P for Phitsanulok), and the fourth the leaf type (F = fresh, D = fallen).

Antibacterial activity

The extracts were evaluated using a modified disc diffusion method following Tendencia (2004). *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Propionibacterium acnes* were first cultured in 2 mL of Mueller–Hinton Broth (MHB) and incubated at 37 °C for 18–24 h. The bacterial suspension was then adjusted to a turbidity equivalent to an optical density of 0.08–0.13 at 625 nm using 0.85% normal saline solution. Afterward, the inoculum was evenly spread onto Mueller–Hinton Agar (MHA) plates and dried for 3–5 min. The extracts were applied to sterile paper discs at a concentration of 500 µg per disc, placed on the inoculated MHA plates and incubated at 37 °C for 18–24 h. The diameters of the inhibition zones were then measured to determine antibacterial activity. Gentamicin (10 µg per disc) was the positive control, while methanol was the negative control.

Gas chromatography-mass spectrometry (GC-MS) analysis

GC–MS analysis was conducted on an Agilent GC coupled with a 5977B MSD. A 1 µL portion of each extract was injected in 50:1 split mode at 250 °C and separated on an HP-5MS column using helium as the carrier gas (1 mL min^{-1}). The oven was held at 50 °C for 3 min, then heated to 250 °C at 10 °C min^{-1} and maintained for 10 min. The MS operated at 260 °C, scanning m/z 50–550 after a 3 min solvent delay, and compounds were identified by comparison with the NIST 20 library.

Statistical analysis

The data were expressed as mean ± standard deviation (SD). Statistical analyses were performed using IBM SPSS Statistics software (version 25.0). Differences among the sample groups were evaluated by one-way analysis of variance (ANOVA), and post hoc comparisons were carried out using Duncan's multiple range test. The probability value of $P < 0.05$ was considered to indicate a statistically significant difference.

Biodiversity analysis

Fresh leaves from 28 teak samples (Table 1) were collected for DNA extraction using the CTAB protocol (Doyle and Doyle, 1990). Leaf tissue was ground in liquid nitrogen, homogenized in CTAB buffer, incubated at 65 °C, and subjected to repeated chloroform: isoamyl alcohol (24:1) extraction. Precipitated DNA was dissolved in TE buffer and checked on 1 % agarose gels stained with ethidium bromide. Thirty SRAP primer combinations from five forward (ME1–ME5) and six reverse (EM1–EM6) primers (Li and Quiros, 2001) were initially tested on six teak accessions (TGJP03, 04, 12, 13, 20, 21). PCR reactions (20 µL) contained 100 ng template DNA, 0.8 µM of each primer, 0.25 mM dNTPs, 2.5 mM $MgCl_2$, 1 U Taq polymerase, and 1× buffer. The thermal program was 94 °C for 3 min; 5 cycles of 94 °C for 1 min, 35 °C for 1 min, 72 °C for 1 min; 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min; and a final extension at 72 °C for 10 min. Amplified products were resolved on 2 % agarose in 1× TBE for 35 min with a 100 bp ladder. SRAP bands were scored as present (1) or absent (0), and similarity coefficients were calculated for UPGMA clustering in NTSYSpc v2.1 to construct the genetic relationship dendrogram.

Table 1. Sample codes, plus-tree/genotype, and collection sites of teak leaves used for genetic diversity analysis

Sample code	Plus-tree/ Genotype	Collection site	Sample code	Plus-tree/ Genotype	Collection site
TGJP01	Chiang Mai	TSRS	TGJP15	Chiang Rai	PSRS
TGJP02	Phrae	TSRS	TGJP16	Chiang Saen	PSRS
TGJP03	Lampang	TSRS	TGJP17	Mae Hong Son	PSRS
TGJP04	Sukhothai	TSRS	TGJP18	Tak	PSRS
TGJP05	Khonkaen	TSRS	TGJP19	Siammint teak	RSPG
TGJP06	Chiang Rai	TSRS	TGJP20	Siammint teak	RSPG
TGJP07	Chiang Saen	TSRS	TGJP21	Mahesak teak	RSPG
TGJP08	Mae Hong Son	TSRS	TGJP22	Siammint teak	Suphan Buri
TGJP09	Tak	TSRS	TGJP23	Mahesak teak	Suphan Buri
TGJP10	Chiang Mai	PSRS	TGJP24	unknown	Sukhothai
TGJP11	Phrae	PSRS	TGJP25	unknown	Kamphaeng Phet
TGJP12	Lampang	PSRS	TGJP26	unknown	Bangkok
TGJP13	Sukhothai	PSRS	TGJP27	unknown	Bangkok
TGJP14	Khonkaen	PSRS	TGJP28	unknown	Bangkok

Note: [1]TSRS = Thongphaphum Silviculture Research Station, Kanchanaburi
[2]PSRS = Phitsanulok Silviculture Research Station, Phitsanulok
[3]RSPG = Plant Genetic Conservation Project Under the Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindhorn

Results

Paper disc diffusion tested twenty liquid–liquid extraction fractions for antibacterial activity against *P. acnes*, *S. aureus*, and *S. epidermidis* (500 µg/disc). Gentamicin (10 µg/disc, positive control) produced inhibition zones of 33.04, 22.78, and 14.50 mm for *S. epidermidis*, *S. aureus*, and *P. acnes*, respectively. Hexane and dichloromethane fractions inhibited all three bacteria (0–14.72 mm), while ethyl acetate fractions inhibited only *P. acnes* and *S. aureus* (0–9.50 mm); n-butanol and aqueous fractions were inactive (Table 2). Fresh leaf extracts from the Thongphaphum silviculture research station (PTF) showed the most potent inhibition, with dichloromethane fractions most active, followed by hexane. Activity was generally higher in fresh than fallen leaves and Thongphaphum than in Phitsanulok samples (Figures 1A–F).

Column chromatography of hexane (H) and dichloromethane (D) extracts from fresh (PTF) and fallen (PTD) Phrae teak leaves yielded 12, 6, 7, and 8 sub-fractions of HPTF, HPTD, DPTF, and DPTD, respectively, as confirmed by normal-phase TLC. Sub-fractions 3–4 of the hexane extract (Table 3) and sub-fractions 2–4 of the dichloromethane extract (Table 4) showed potent antibacterial activity, except that fallen-leaf fractions failed to inhibit *P. acnes*; overall, fallen leaves inhibited *S. aureus* and *S. epidermidis* more effectively than fresh leaves.

GC–MS analysis identified known chemical constituents in the selected sub-fractions: 8 and 6 compounds in the hexane fractions from fresh leaves (HPTF3 and HPTF4), 21 and 7 compounds from fallen leaves (HPTD3 and HPTD4), 3 and 13 compounds in the dichloromethane fractions from fresh leaves (DPTF3 and DPTF4), and 5 and 8 compounds from fallen leaves (DPTD3 and DPTD4). Five noteworthy compounds with potential antibacterial activity were detected: hexadecanoic acid methyl ester, phenol, 2,4-bis(1,1-dimethylethyl), stigmasterol, spathulenol, and loliolide.

Genetic diversity analysis using SRAP markers

A total of 30 SRAP primer combinations were initially screened using six teak samples, from which six primer pairs (ME1/EM3, ME2/EM4, ME2/EM5, ME3/EM2, ME5/EM1, and ME5/EM5) producing clear polymorphic bands were selected. These six primers were subsequently used to evaluate the genetic diversity of 28 samples. Sixty DNA bands were amplified, ranging from 100 to 1500 bp. Among these, 40 bands were polymorphic, representing 66.67% of the total bands (Table 5).

Table 2. Antibacterial activity of teak leaf fractions (500 µg/disc) obtained by liquid–liquid extraction against *P. acnes*, *S. aureus*, and *S. epidermidis*

Organisms	Fractions	Inhibitory Zone (mm)				
		Hexane	Dichloro methane	Ethyl Acetate	Butanol	Water
P. acnes	PTF	13.95^{a}±0.40	13.86^{ab}±0.50	7.21^{bc}±0.44	0.00	0.00
	PTD	8.46^{d}±0.11	7.90^{f}±0.44	8.68^{a}±0.92	0.00	0.00
	PPF	7.66^{e}±0.35	8.19^{f}±0.77	0.00	0.00	0.00
	PPD	0.00	7.55^{f}±0.57	9.50^{a}±0.91	0.00	0.00
S. aureus	PTF	11.86^{b}±0.09	12.52^{cd}±0.93	0.00	0.00	0.00
	PTD	10.13^{c}±1.46	11.36^{d}±0.37	7.64^{b}±0.23	0.00	0.00
	PPF	7.66^{e}±0.35	7.39^{f}±0.89	0.00	0.00	0.00
	PPD	7.34^{e}±0.28	0.00	6.79^{c}±0.08	0.00	0.00
S. epidermidis	PTF	12.14^{b}±0.78	14.72^{a}±1.61	0.00	0.00	0.00
	PTD	11.19^{b}±0.52	13.26^{bc}±0.95	0.00	0.00	0.00
	PPF	7.97^{de}±0.23	10.04^{e}±0.49	0.00	0.00	0.00
	PPD	0.00	7.59^{f}±0.53	0.00	0.00	0.00

Note: Values are mean ± SD of inhibition zones; different letters (a–f) indicate significant differences (DMRT, $p < 0.05$).

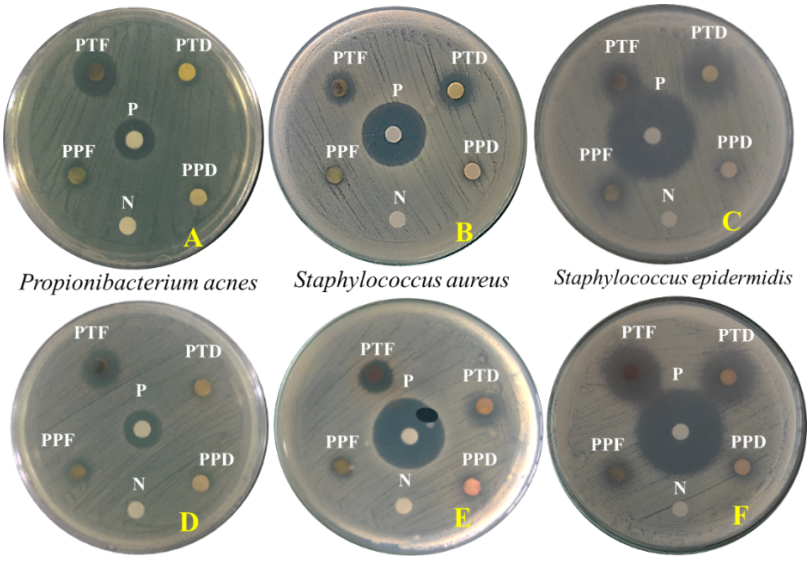

Figure 1. Antibacterial activity of teak leaf extracts against (A-D) *P. acnes*, (B-E) *S. aureus*, and (C-F) *S. epidermidis*: hexane (top) and dichloromethane (bottom) fractions from Phrae plus trees. P = positive control (gentamicin 10 µg/disc); N = negative control (methanol)

Table 3. Antibacterial activity of teak leaf sub-fractions (500 µg/disc) from hexane extract of Phrae plus trees at Thongphaphum station, purified by column chromatography, and gentamicin (10 µg/disc) against *P. acnes*, *S. aureus*, and *S. epidermidis*

Sub-fractions from hexane	Inhibitory Zone (mm)					
	P. acnes		***S. aureus***		***S. epidermidis***	
	HPTF	**HPTD**	**HPTF**	**HPTD**	**HPTF**	**HPTD**
F1	0.00	0.00	0.00	0.00	6.45^{d}±0.65	0.00
F2	0.00	0.00	11.20^{b}±0.31	0.00	11.46^{b}±1.81	0.00
F3	8.55^{b}±0.61	0.00	12.47^{a}±0.86	14.97^{a}±1.46	15.14^{a}±2.01	16.02^{a}±2.25
F4	10.60^{a}±0.65	0.00	12.23^{a}±0.30	9.29^{b}±1.35	15.32^{a}±0.24	11.52^{b}±1.05
F5	9.98^{a}±0.25	ND	11.14^{b}±0.17	ND	11.03^{b}±0.71	ND
F6	0.00	ND	0.00	ND	0.00	ND
F7	9.95^{a}±0.63		0.00		0.00	
F8	0.00		0.00		0.00	
F9	ND		ND		ND	
F10	0.00		0.00		0.00	
F11	0.00		7.47^{c}±0.00		8.48^{c}±0.00	
F12	ND		ND		ND	
Gentamicin	15.70±0.01		24.14±0.06		30.42±0.41	

Note: ND = Not detected; values are mean ± SD of inhibition zones, and different letters (a–d) indicate significant differences (DMRT, $p < 0.05$).

Table 4. Antibacterial activity of teak leaf sub-fractions (500 µg/disc) from dichromethane extract of Phrae plus trees at Thongphaphum station, purified by column chromatography, and gentamicin (10 µg/disc) against *P. acnes*, *S. aureus*, and *S. epidermidis*

Sub-fractions from dichromethane	Inhibitory Zone (mm)					
	P. acnes		***S. aureus***		***S. epidermidis***	
	DPTF	**DPTD**	**DPTF**	**DPTD**	**DPTF**	**DPTD**
F1	0.00	0.00	0.00	0.00	0.00	0.00
F2	8.99^{b}±0.15	0.00	10.39^{b}±0.81	10.41^{c}±0.04	11.65^{b}±0.71	8.66^{c}±0.70
F3	12.78^{a}±0.40	0.00	10.04^{b}±0.34	13.27^{a}±0.27	12.72^{b}±1.18	15.69^{a}±1.56
F4	8.14^{c}±0.38	0.00	11.69^{a}±0.68	13.03^{b}±0.13	16.63^{a}±0.80	13.97^{b}±1.82
F5	0.00	0.00	7.90^{c}±0.17	7.73^{d}±0.24	0.00	0.00
F6	0.00	0.00	7.22^{c}±0.70	0.00	7.71^{c}±0.70	0.00
F7	ND	0.00	ND	0.00	ND	0.00
F8		0.00		0.00		0.00
Gentamicin	15.18±0.11		23.64±0.34		30.76±0.1	

Note: ND = Not detected; values are mean ± SD of inhibition zones, and different letters (a–d) indicate significant differences (DMRT, $p < 0.05$).

The UPGMA dendrogram generated from SRAP data revealed similarity coefficients ranging from 0.67 to 1.00 (Figure 2). At a coefficient of 0.84, the 28 teak samples were grouped into four main clusters, with Cluster 1 further divided

into two subclusters. However, the grouping did not clearly separate trees by clone or planting location. For example, TGJP19 (Siammint variety from the RSPG) and TGJP26 (unknown variety from Bangkok) clustered closely with other samples, while TGJP05 (Khon Kaen plus tree from Thongphaphum Research Station) and TGJP15 (Chiang Rai plus tree from Phitsanulok Research Station), originating from different regions, also exhibited high genetic similarity.

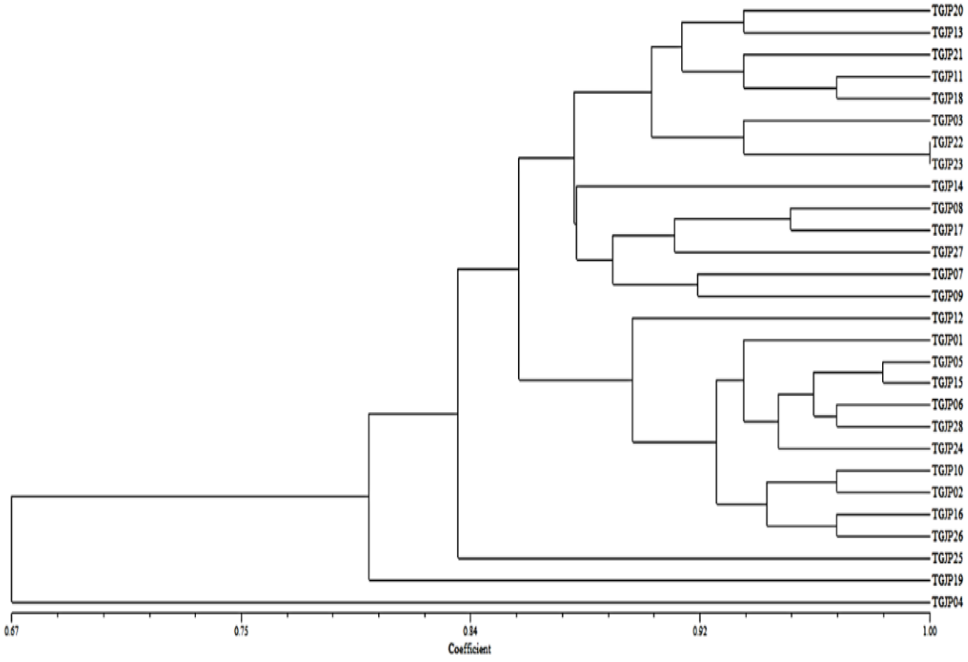

Figure 2. Dendrogram showing the genetic relationships among 28 teak plus trees based on SRAP marker analysis. The clustering was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) to illustrate genetic distances

Table 5. SRAP primer profile, number of DNA bands, polymorphic bands, and percentage of polymorphism for 28 samples

Primer combination	Total DNA bands	Polymorphic bands	% Polymorphism
ME1/EM3	9	7	77.78
ME2/EM4	11	9	81.82
ME2/EM5	14	7	50.00
ME3/EM2	10	6	60.00
ME5/EM1	8	6	75.00
ME5/EM5	8	5	62.50
Total / Average	60	40	66.67

Discussion

Previous research by Phonmakham *et al*. (2018) demonstrated that the identity of the plus tree significantly influenced the presence of bioactive compounds in teak. Among the tested samples, crude methanol extracts from the Phrae plus tree showed the most potent antibacterial activity. However, the effects of leaf stage (fresh vs. fallen) and planting site (Thongphaphum vs. Phitsanulok Silviculture Research Station) remained unclear. To address this, 20 fractions derived from four Phrae plus tree leaf samples—fresh and fallen leaves collected from both Thongphaphum and Phitsanulok stations and sequentially extracted with five solvents—were evaluated for factors affecting bioactivity, focusing specifically on antibacterial activity. Dichloromethane fractions showed the most potent antibacterial activity, followed by hexane and ethyl acetate, with extracts from Thongphaphum exhibiting greater effects than those from Phitsanulok. Therefore, column chromatography was used to select only hexane and dichloromethane extracts from fresh and fallen Phrae plus tree leaves at Thongphaphum for purification and chemical analysis. Column chromatography of hexane and dichloromethane extracts from fresh and fallen Phrae teak leaves yielded multiple sub-fractions, with hexane sub-fractions 3–4 and dichloromethane sub-fractions 2–4 showing strong antibacterial activity, and

fallen leaves were generally more effective against *S. aureus* and *S. epidermidis* but ineffective against *P. acnes.* This is consistent with the antibacterial activity reported by Purushotham and Sankar (2013) and Lanka and Parimala (2017).

SRAP analysis of 28 teak samples revealed 66.7% polymorphic bands, indicating substantial genetic variation. The UPGMA dendrogram grouped the samples into four main clusters. Yet, clustering did not clearly correspond to clone identity or planting location, as exemplified by TGJP05 (Khon Kaen) and TGJP15 (Chiang Rai), which exhibited high similarity despite originating from different regions. This suggests significant gene flow or shared ancestry among the plus trees. These results are not consistent with previous molecular studies. In India, RAPD and ISSR analyses revealed broad genetic diversity among teak provenances, with little correspondence between genetic clustering and geographic origin (Narayanan *et al*., 2007), but are consistent with the findings of Thakor *et al*. (2019), in which SRAP markers detected a wide range of similarity coefficients (0.11–0.92). This is consistent with RAPD and SSCP analyses of natural teak populations in Thailand, which revealed high polymorphism and multiple genetic groups (Changtragoon and Szmidt, 2000; Pongkrawee and Volkaert, 2012). These findings indicate that teak exhibits substantial intraspecific variation, while geographic origin may have limited influence on genetic clustering in cultivated plus trees.

Considering both bioactivity and genetic diversity, the study indicates that while the identity of the plus tree affects bioactivity and the concentration of key compounds, SRAP analysis revealed relatively moderate genetic diversity among teak samples, and no apparent clustering by the plus tree or planting location. This suggests that the bioactive compounds in teak leaf extracts are likely similar across plus trees. However, their concentrations may vary depending on the cultivation site, due to differences in environment, climate, temperature, light, rainfall, and soil minerals. Although antibacterial activity was similar between fresh and fallen leaves, the purified extract from fallen leaves exhibited more substantial antibacterial effects, despite showing no activity against *P. acnes*. Therefore, for practical applications, fallen leaves are preferable since collecting them does not affect tree growth, and they are a natural by-product of teak cultivation, the primary purpose of which is timber production.

Acknowledgements

This work was supported by King Mongkut's Institute of Technology Ladkrabang (KMITL), Grant No. 2562-01-05-29. The author gratefully acknowledges Dr. Saroj Wattanasuksakul of the Forest Research and Development Bureau, Royal Forest Department, and the Thongphaphum and Phitsanulok Silviculture Research Stations staff for their assistance.

Conflicts of interest

The authors declare no conflict of interest.

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(Received: 25 September 2025, Revised: 13 January 2026, Accepted: 26 February 2026)