



Total phenolic and flavonoid contents, bioactivity, and cytotoxicity of teak leaves and application in functional plant-based cookies

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ABSTRACT

Background: Teak (*Tectona grandis*) leaves are rich in bioactive compounds, particularly phenolics and flavonoids, which exhibit diverse biological activities. However, their utilization in functional foods remains limited. This study assessed the phytochemical composition, biological activities, and cytotoxicity of teak leaf extract and developed gluten-free functional cookies enriched with teak leaf powder. Modified cassava (mocaf) and almond flour were used as gluten-free ingredients to support digestive health.

Methods: Teak leaf extract was analyzed for total phenolic and flavonoid contents and evaluated for its antioxidant (DPPH assay), anti-inflammatory (MIF inhibitory assay), antidiabetic (α -glucosidase inhibitory assay), tyrosinase inhibitory assay, and cytotoxic (MTT assay) activities. Teak leaf powder was then incorporated into plant-based cookie formulations prepared with mocaf and almond flour. The cookies were characterized by dietary fiber content, textural

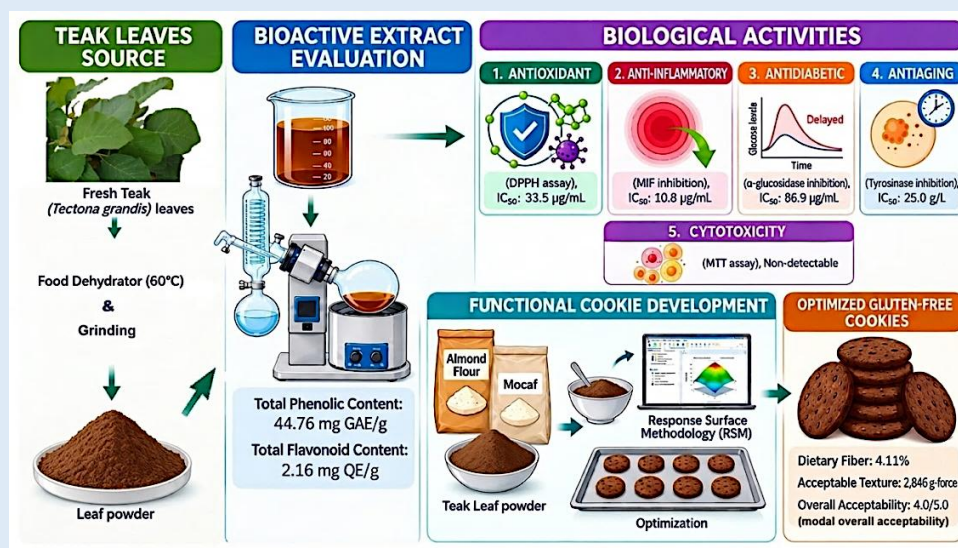
properties, and overall sensory acceptability. The formulation was optimized using response surface methodology (RSM) with Design-Expert software based on three response variables.

Results: Quantitative phytochemical analyses revealed the total phenolic and flavonoid contents of 44.76 ± 1.91 mg GAE/g and 2.16 ± 0.42 mg QE/g, respectively. The extract showed antioxidant activity with an IC_{50} of 33.5 ± 2.6 μ g/mL, anti-inflammatory activity with an IC_{50} of 10.8 ± 3.1 μ g/mL, antidiabetic activity with an IC_{50} of 86.9 ± 1.1 μ g/mL, and antiaging activity with an IC_{50} of $25,000 \pm 800$ μ g /L. No cytotoxic effects were observed. The optimal formulation, consisting of 40% moca and 60% almond flour, yielded gluten-free cookies with predicted enhanced dietary fiber content ($4.11 \pm 0.01\%$), acceptable texture ($2,846.0 \pm 17.9$ g-force), and overall sensory acceptability of 4.0 out of 5.0. In the experimental sensory profile, category 4 was the modal overall acceptability response.

Conclusion: The findings support further investigation of teak leaf powder as a potential bioactive compound with promising biological activities and demonstrate effective incorporation into gluten-free cookie formulations, while acknowledging that further studies are necessary to establish food safety and possible chronic disease prevention.

Novelty of the Study: This study demonstrates the potential valorization of underutilized teak leaf biomass as a bioactive ingredient for gluten-free functional food development. It integrates the evaluation of multiple in vitro bioactivities of teak leaf extract with its incorporation into moca- and almond flour-based cookies, using response surface methodology to optimize the formulation. The optimized product achieved a favorable balance of dietary fiber content, texture, and sensory acceptability while retaining the potential functional relevance of teak leaf bioactives.

Keywords: Teak leaves, phytochemical analyses, biological activities, plant-based cookies, response surface methodology.



Graphical Abstract: Total phenolic and flavonoid contents, bioactivity, and cytotoxicity of teak leaves and application in functional plant-based cookies

INTRODUCTION

The global rise in chronic non-communicable diseases, including diabetes and chronic inflammatory conditions, has intensified interest in functional foods as dietary strategies for disease prevention and health promotion. These conditions are closely associated with lifestyle factors and oxidative stress, driving the need for dietary interventions that promote health and help prevent disease. Functional foods are formulated with bioactive compounds, such as polyphenols, flavonoids, and other phytochemicals, that provide physiological benefits beyond basic nutrition while remaining safe and effective at intake levels [1-7]. As a result, the development of functional food formulations has become an important focus in nutrition research and the food industry.

Within this context, Indonesia, one of the world's richest centers of biodiversity, represents a valuable source of underutilized plant resources with significant potential for functional food development. However, many endemic and indigenous plant species remain insufficiently characterized in terms of their phytochemical composition, biological activities, safety, and suitability for food applications. This knowledge gap limits their utilization as functional ingredients and value-added products. The development of functional foods requires systematic progression from the identification and characterization of bioactive compounds to the evaluation of their biological activity, safety, formulation potential, and eventual translation into functional food products. Such an integrated approach is essential to ensure that promising plant resources are supported by sufficient scientific evidence before being developed as functional food ingredients [2]. Therefore, systematic research is essential to unlock the potential of these underexplored species and support their integration into sustainable and evidence-based functional food development.

Among these species, teak (*Tectona grandis*) stands out as a particularly promising candidate, especially its

leaves, which have been traditionally used in East Java as natural food wrappers and colorants. This longstanding use reflects their cultural acceptance and calls for studies to test their safety for food applications. Recent studies have demonstrated that teak leaves are rich in phytochemicals, particularly flavonoids and phenolic compounds, which exhibit antioxidant, anti-inflammatory, antidiabetic, antiaging effects, and other therapeutic potentials, providing a rationale for further investigation of teak leaves as a source of functional bioactives [3-4,7-12].

Further supporting their potential, previous studies have identified numerous bioactive compounds from methanolic extracts of teak leaves. Among these, verbascoside and gallic acid exhibited antioxidant activities comparable to or greater than ascorbic acid. In addition, teak leaf extracts have been reported to enhance glucose uptake in adipocytes, suggesting their potential to improve glycemic regulation and support metabolic health [3-4,11]. Collectively, these findings highlight teak leaves as a multifunctional bioresource with strong potential for development into functional food products.

Antioxidant activity is a critical parameter underpinning the preventive role of functional foods against oxidative stress, a major contributor to the development of chronic diseases. Teak leaf extracts have demonstrated *in vitro* antioxidant activity [10,13], with IC₅₀ values below 50 µg/mL, meeting the criteria for strong antioxidant activity in food science [14]. Furthermore, bioactive constituents such as anthraquinones and phenolics have been shown to inhibit pro-inflammatory cytokines, including TNF-α and IL-6, suggesting additional systemic health benefits [3-4, 15]. Ethanol extracts exhibit low cytotoxicity [16], supporting their potential for food applications [17]. Nevertheless, establishing safe dosage levels remains essential, as toxicological studies indicate that high doses of aqueous extracts (e.g., 40 mg/kg body weight) may

induce histopathological changes in animal models [18].

To address these gaps, this study evaluated the phytochemical composition, various *in vitro* biological activities, and cytotoxicity of teak leaf extract. In addition, teak leaf powder was incorporated into gluten-free plant-based cookies, and the formulation was optimized using response surface methodology (RSM) to achieve desirable dietary fiber content, textural properties, and overall sensory acceptability. This integrated approach is consistent with the Functional Food Center (FFC) 17-Step Framework for functional food development, in which characterization of bioactive compounds, evaluation of biological effects and safety, and optimization of the final food formulation are interconnected steps toward the development of evidence-based functional foods [2]. Establishing these preliminary steps provides the necessary scientific foundation before progressing to safety confirmation, clinical studies, and regulatory recognition. Therefore, this study provides a scientific basis for developing teak leaves as a safe and functional food ingredient while supporting the sustainable utilization of Indonesia's underexplored plant resources.

MATERIALS AND METHODS

Sample Preparation and Extraction: Fresh teak (*Tectona grandis*) leaves were collected from Kebontungul Village, East Java, Indonesia, and taxonomically authenticated by the Faculty of Biotechnology, University of Surabaya. The collected leaves were air-dried to remove surface moisture and subsequently dehydrated at 60°C using a food dehydrator to preserve thermolabile phytochemicals. The dried leaves were ground into a fine powder and sieved through a 40-mesh screen to obtain a uniform particle size. For phytochemical extraction, 10 g of powdered sample was macerated in 100 mL of 96% (v/v) ethanol at 25°C for 72 h with intermittent agitation. The extract was filtered through Whatman No. 1 filter paper and concentrated under reduced pressure using a rotary evaporator at 40°C. The concentrated extract was stored in amber glass bottles at 4°C in the refrigerator until further analysis.

Total Phenolic and Total Flavonoid Content: Total phenolic content (TPC) was determined using the Folin–Ciocalteu method, with gallic acid as the reference standard [19–20]. 500 µL of leaf extract was mixed with 10 mL of distilled water and 0.5 mL of Folin–Ciocalteu reagent. Following a 5-min incubation, 8 mL of 7.5% sodium carbonate solution was incorporated into the mixture. The reaction was then left to proceed in the dark for 2 h. Absorbance was measured spectrophotometrically using a spectrophotometer at 765 nm, and results were calculated from a gallic acid reference curve and expressed as mg gallic acid equivalents (GAE)/g of extract. Total flavonoid content (TFC) was determined using the aluminum chloride (AlCl₃) colorimetric method, with quercetin as the reference standard [20]. To perform the assay, 1 mL of leaf extract was treated with 1 mL of 2% aluminum chloride. The resulting mixture was then held at 25°C for a 15-min incubation period. Absorbance was measured spectrophotometrically at 430 nm, and TFC was calculated from the quercetin reference curve and expressed as mg quercetin equivalents (QE)/g extract.

Antioxidant Assay: The antioxidant activity of teak leaf extract was evaluated using the DPPH radical scavenging assay [21–24]. Briefly, 3 mL of 0.3 mM DPPH solution was combined with 100 µL of leaf extract at various concentrations (12.5–200.0 µg/mL). The mixture was incubated in the dark for 30 min, and the absorbance was measured spectrophotometrically at 517 nm. The percentage of DPPH radical scavenging activity was calculated relative to the DPPH control, and the IC₅₀ value was determined using non-linear regression analysis. The test was carried out in triplicate.

Anti-inflammatory Assay: Anti-inflammatory activity was evaluated by measuring MIF tautomerase inhibition

using recombinant His-tagged MIF expressed in *E. coli* BL21 (DE3) and purified using Ni-NTA affinity chromatography. The assay, conducted in triplicate, was performed according to Kok et al. (2018) with minor modifications. Briefly, 45 μ L recombinant MIF (20.6 μ g/mL) was incubated with 5 μ L teak leaf extract that has been diluted using DMSO (1.6–100.0 μ g/mL), and the reaction was initiated by adding 50 μ L 4-hydroxyphenylpyruvate (4-HPP, 1 M) in ammonium acetate buffer (pH 6.0). Changes in absorbance at 306 nm were recorded kinetically at 37°C using a microplate reader. A reaction mixture without enzyme served as the negative control, and IC₅₀ values were calculated from the inhibition of tautomerase activity [25].

Antidiabetic Assay: Antidiabetic activity was evaluated using α -glucosidase inhibitory assay. The assay was assessed in triplicate. Each well contained 50 μ L of 0.1 M phosphate-buffered saline (PBS, pH 6.9), 10 μ L of α -glucosidase solution (1 ppm), and 20 μ L of either sample (31.3–500.0 μ g/mL) or standard. Following incubation at 37°C for 15 min, 20 μ L of 4-nitrophenyl- α -D-glucopyranoside (4-NPG) was added to initiate the enzymatic reaction, and the mixture was incubated for an additional 20 min. The reaction was terminated by adding 50 μ L of 0.1 M sodium carbonate (Na₂CO₃), and the absorbance was measured at 405 nm using a microplate reader [26].

The percentage inhibition was calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{(\text{Absorbance of blank} - \text{Absorbance of sample})}{\text{Absorbance of blank}} \times 100$$

Tyrosinase Inhibitory Assay: Tyrosinase inhibitory assay was conducted by evaluating tyrosinase inhibitory activity [27–28]. The assay was assessed in triplicate. A total of 100 mg of the sample was dissolved in 1 mL of DMSO to prepare 100 mg/mL stock solution and,

subsequently diluted with 0.1 M phosphate-buffered saline (PBS, pH 6.8) to the desired concentrations (1,500–25,000 μ g/mL). Each reaction mixture contained 40 μ L of L-DOPA (5 mM), 40 μ L of sample or standard, 160 μ L of PBS, and 40 μ L of mushroom tyrosinase (250 U/mL). The microplate was then covered with aluminum foil to protect from light and incubated at 25°C for 15 min. After incubation, absorbance was measured at 490 nm using a microplate reader.

Tyrosinase inhibition was calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{(\text{Absorbance of blank} - \text{Absorbance of sample})}{\text{Absorbance of blank}} \times 100$$

Cytotoxicity Assay: Cytotoxicity was evaluated using the MTT assay. The assay was assessed in triplicate. Vero cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) to support optimal cell growth and viability. Adherent or centrifuged non-adherent cell cultures were refreshed with 100 μ L of medium per well, followed by the addition of 10 μ L of 12 mM MTT stock solution and subsequent incubation at 37°C for 2–4 h. The cells were then treated with varying concentrations of teak leaf extract ranging from 31.3 to 1,000.0 μ g/mL; medium-only wells containing MTT served as the negative control. After treatment, 100 μ L of sodium dodecyl sulfate–hydrochloric acid (SDS–HCl) solution was introduced directly to each well, incubated in a humidified chamber at 37°C for 4–18 h, mixed thoroughly, and analyzed for absorbance at 570 nm, and cell inhibition (%) was plotted against extract concentration to establish the dose–response relationship and assess the cytotoxicity of the extract [29].

Cookie Formulation and Optimization: Plant-based gluten-free cookies were formulated by incorporating 1.5% teak leaf powder into composite flour mixtures consisting

of modified cassava flour (mocaf) and almond flour. Cookie dough was baked at 170°C for 10 min and cooled to 25°C. Formulation optimization was performed using RSM [30] with a D-optimal mixture design implemented in Design-Expert Version 13. A polynomial model analysis was conducted to determine the recommended predictive model. The statistical significance of the generated model was evaluated using analysis of variance (ANOVA) at a significance threshold of $p < 0.05$. For the significant model, the influence of the mocaf-to-almond flour ratio on dietary fiber content, texture, and overall sensory acceptability was evaluated using RSM to determine the optimal formulation.

The optimal formulation was then confirmed by comparing the RSM-predicted responses of the selected formulation with the experimental sensory profiling to confirm the reliability and reproducibility of the optimal formulation. The sensory profiling was conducted using 69 semi-trained panelists, who assessed crispness, color, aroma, mouthfeel, taste, aftertaste, and overall sensory acceptability using a five-point hedonic scale. The

proximate and microbiological analysis were subsequently performed to verify the nutritional value and safety.

RESULTS

Total Phenolic and Total Flavonoid Content: The TPC of the teak leaf extract, determined by the Folin–Ciocalteu method with gallic acid as a reference standard, was 44.76 ± 1.91 mg GAE/g. This indicates that teak leaves are a rich source of phenolic compounds, which may contribute to their antioxidant activity and potential application as a functional food ingredient. The TFC was (2.16 ± 0.42) mg QE/g, confirming the presence of flavonoid compounds that may contribute to the biological activities of the extract.

Antioxidant Assay: The teak leaf extract exhibited concentration-dependent DPPH radical scavenging activity (Figure 1). The percentage of inhibition increased with increasing extract concentrations, and the IC_{50} value was determined to be 33.5 ± 2.6 μ g/mL, confirming the antioxidant activity of the teak leaf extract.

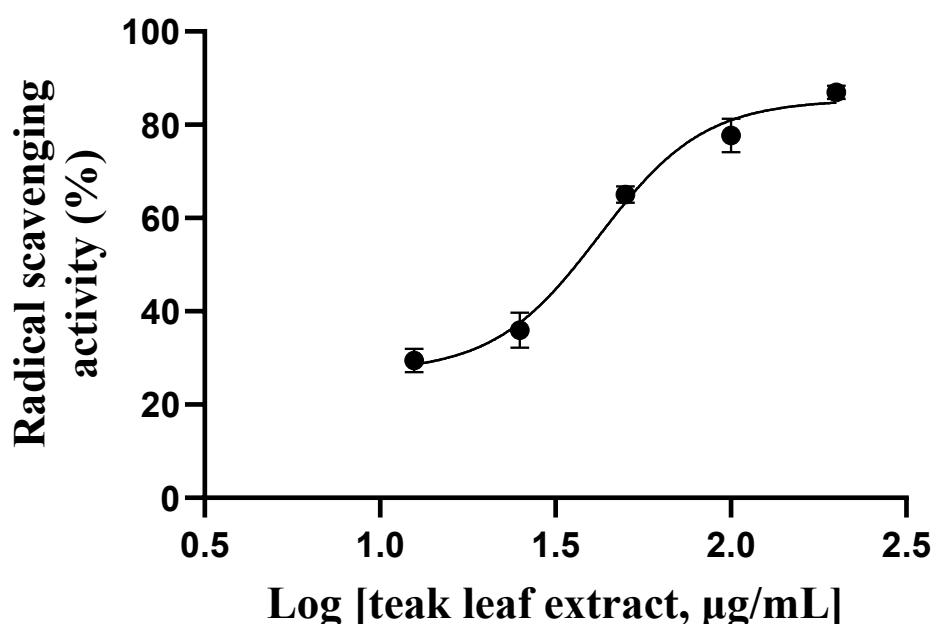


Figure 1. Radical scavenging activity by teak leaf extract. The assay was assessed in triplicate.

Anti-inflammatory Assay: The extract exhibited dose-dependent inhibition against MIF tautomerase activity. Higher concentrations of the extract resulted in greater suppression of enzyme activity, as reflected by reduced absorbance values (Figure 2). The IC_{50} value was $10.8 \pm$

$3.1 \mu\text{g/mL}$, indicating inhibitory activity against MIF tautomerase and suggesting that teak leaf extract may have potential to modulate MIF-related inflammatory pathways.

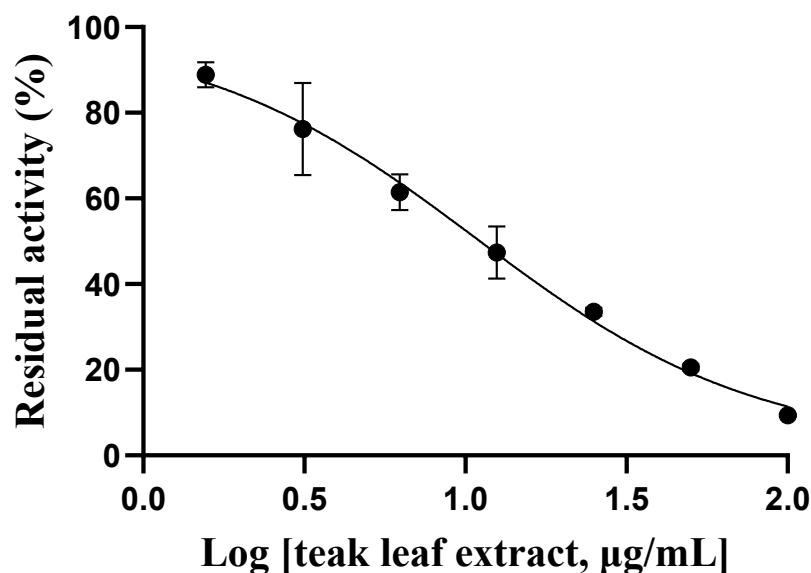


Figure 2. Inhibition of MIF activity by teak leaf extract. The assay was assessed in triplicate, with negligible standard deviations at some points.

Antidiabetic Assay: The α -glucosidase assay evaluated inhibitory activity based on the inhibition of 4-NPG hydrolysis; greater inhibition yields less yellow color. The

teak leaf extract showed an IC_{50} of $86.9 \pm 1.1 \mu\text{g/mL}$ in the α -glucosidase inhibitory assay (Figure 3).

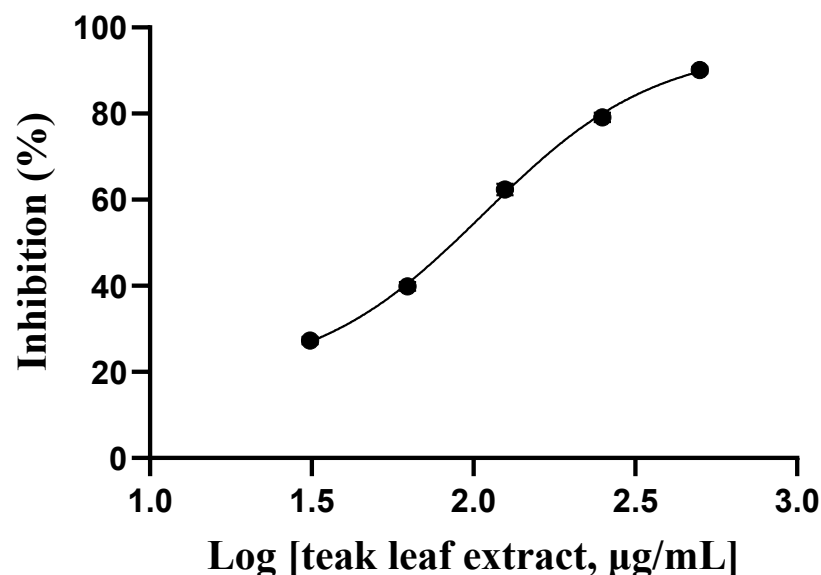


Figure 3. Inhibition of α -glucosidase by teak leaf extract. The assay was conducted in triplicate, with negligible standard deviations that are not visually discernible.

Tyrosinase Inhibitory Assay: The tyrosinase inhibitory assay evaluated the anti-melanogenic potential by measuring the inhibition of dopachrome formation from

L-DOPA. The teak leaf extract exhibited an IC_{50} value of $25,000 \pm 800 \mu\text{g/mL}$, indicating relatively weak tyrosinase inhibitory activity (Figure 4).

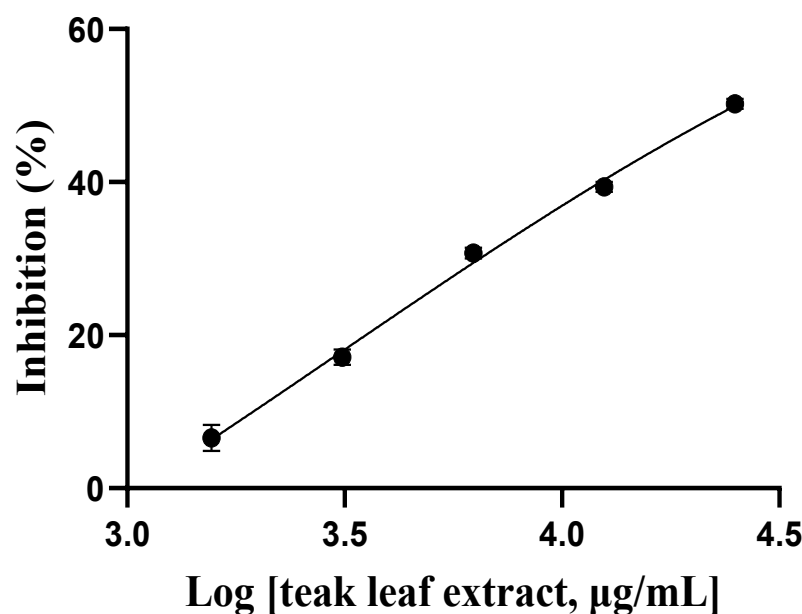


Figure 4. Inhibition of tyrosinase activity by teak leaf extract. The assay was run in triplicate, with negligible standard deviations that are not visually discernible.

Cytotoxicity Assay: No 50% inhibition of Vero-cell metabolic activity was observed within the tested concentration range (31.3 – $1,000.0 \mu\text{g/mL}$); therefore, an

IC_{50} could not be determined under the experimental conditions (Figure 5).

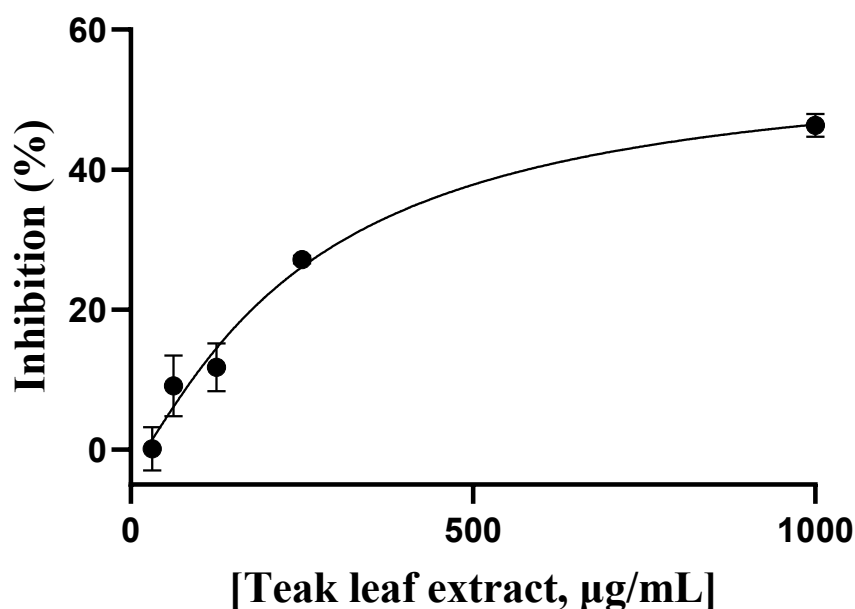


Figure 5. Inhibition of cell metabolic activity by teak leaf extract. The assay was assessed in triplicate.

Cookie Formulation and Optimization: Among the polynomial models evaluated, the quadratic model was selected because the addition of quadratic terms significantly improved model fit (sequential $p = 0.0018$),

whereas further expansion to cubic ($p = 0.1833$) or quartic ($p = 0.3225$) models did not result in significant improvement (Table 1).

Table 1. Polynomial Model Analysis

Source	Sequential p-value	Lack of Fit p-value	Adjusted R ²	Predicted R ²	Remarks
Linear	< 0.0001	0.0092	0.9436	0.9299	
Quadratic	0.0018	0.2582	0.9755	0.9700	Recommended
Cubic	0.1833	0.3225	0.9776	0.9657	
Quartic	0.3225		0.9778		

ANOVA results showed that both the linear mixture effect ($p < 0.0001$; $F = 503.11$) and the interaction between mocaf and almond flour (AB; $p = 0.0018$; $F = 16.65$) significantly affected the combined responses. In contrast, the lack-of-fit test was not significant ($p =$

0.2502), supporting the suitability of the model for describing the experimental data (Table 2).

The RSM-optimized formulation, containing 40% mocaf and 60% almond flour, achieved a desirability value of 0.985 (Table 3).

Table 2. Analysis of Variance (ANOVA)

Source	Sum of Squares	df	Mean Square	F-value	p-value	Remarks
Model	0.2724	2	0.1362	259.88	< 0.0001	Significant
Linear mixture	0.2637	1	0.2637	503.11	< 0.0001	
AB	0.0087	1	0.0087	16.65	0.0018	
Residual	0.0058	11	0.0005			
Lack of Fit	0.0015	2	0.0007	1.58	0.2502	Not significant
Pure Error	0.0043	9	0.0005			
Total	0.2782	13				

Table 3. Optimization Results Based on the Desirability Function

No	Mocaf flour (%)	Almond flour (%)	Texture (g-force)	Dietary fiber (%)	Overall sensory acceptability	Desirability	Remarks
1	40	60	2,846.0	4.11	4.0	0.985	Optimal
2	60	40	4,167.0	3.74	3.9	0.540	
3	50	50	3,240.0	3.88	3.2	0.315	

Confirmation of the Optimal Desirable Formulation, Proximate and Microbiological Analysis: Sensory profiling ($n = 69$) showed high crispiness (category 5), an appealing light brown color (category 4), mild teak aroma and smooth texture (category 3), pleasant sweetness

(category 4), and acceptable slight astringency (category 3). The overall sensory acceptability score (category 4) indicated modal overall acceptability of the optimized formulation (Table 4).

Table 4. Sensory profile of teak leaf plant-based cookies (n = 69 panelists)

Category	Crispiness	%	Color	%	Aroma	%	Texture	%	Taste	%	Aftertaste	%	Overall Sensory Acceptability	%
1	Not crispy	2.9	Very dark brown (almost black)	5.8	Not typical of teak leaves	7.3	Very coarse	8.7	Very bitter	5.8	Very astringent, unpleasant	8.7	Strongly dislike	8.7
2	Less crispy	7.3	Dark brown	8.7	Slightly untypical	17.4	Coarse	21.7	Bitter	11.6	Slightly astringent, less pleasant	17.4	Dislike	13.0
3	Moderately crispy	12.0	Reddish brown	13.0	Slight characteristics of teak leaves	43.5	Slightly smooth	40.6	Slightly sweet	17.4	Slightly astringent but acceptable	39.1	Fair	14.5
4	Crispy	19.8	Light brown	55.0	Characteristics of teak leaves	21.7	Smooth	17.4	Sweet	50.7	Mildly astringent, pleasant	21.8	Like	52.2
5	Very crispy	58.0	Yellowish brown	17.5	Strong teak-leaf aroma	10.1	Very smooth	11.6	Very sweet	14.5	Slightly astringent but pleasant	13.0	Strongly like	11.6

Proximate analysis of the optimized formulation showed a low moisture content of 2.30 ± 0.04 %, which may contribute to favorable product quality and storage characteristics. The ash content was 1.20 ± 0.12 %, reflecting the mineral composition of the formulation. The cookies contained a relatively high fat content (35.40

± 0.20 %), which may contribute to the texture and energy value, along with moderate protein content (5.34 ± 0.40 %). Carbohydrates (55.76 ± 0.40 %) were the major proximate component, representing the primary source of energy in the formulation (Table 5).

Table 5. Proximate composition of optimized teak leaf plant-based cookies

Proximate Parameter	Value (%), Mean $\pm$ SD
Moisture Content	2.30 ± 0.04
Ash Content	1.20 ± 0.12
Fat Content	35.40 ± 0.20
Protein Content	5.34 ± 0.40
Carbohydrate Content	55.76 ± 0.40

Microbiological analysis showed that the total plate count and yeast/mold counts were below the detection limit (< 10 CFU/g), indicating compliance with safety standards.

DISCUSSION

Total Phenolic and Flavonoid Content of Teak Leaves:

Phenolic and flavonoid compounds are important contributors to the antioxidant activity of teak leaf extract because they can act as electron or hydrogen donors, thereby neutralizing free radicals and terminating oxidative chain reactions. In the present study, the teak leaf extract exhibited a TPC of 44.76 ± 1.91 mg GAE/g extract and a TFC of 2.16 ± 0.42 mg QE/g extract. These findings are consistent with previous studies reporting teak leaves as a source of polyphenolic compounds [3,13]. The relatively high TPC may contribute to the observed radical scavenging activity, as phenolic compounds, including quercetin, gallic acid, and related phenolic derivatives, are known to possess antioxidant properties.

These findings highlight the potential of teak leaves as a natural source of antioxidant compounds for functional food applications. The incorporation of teak leaf powder into plant-based cookies may contribute bioactive phenolic and flavonoid compounds to the final

product. In addition, these compounds may have the potential to influence oxidative stability in food systems. Such properties could be particularly relevant to high-fat bakery products, in which lipid oxidation is an important mechanism of quality deterioration during storage [31].

Antioxidant Activity: The DPPH radical scavenging assay revealed that teak leaf extract exhibited appreciable antioxidant activity, with an IC_{50} value of 33.5 ± 2.6 μ g/mL (Figure 1). Although this activity was lower than that of vitamin C, which was used as the reference (3.4 ± 0.6 μ g/mL), the extract demonstrated substantial radical-scavenging activity for a crude plant extract. Based on the classification criteria reported in the literature, extracts with IC_{50} values below 50 μ g/mL are generally considered to exhibit strong antioxidant activity [32]. Thus, the present findings support the potential of teak leaf extract as a natural source of antioxidant compounds.

The observed antioxidant activity may be associated with the presence of phenolic compounds, flavonoids, anthocyanins, and other secondary metabolites previously identified in teak leaves [21,33]. These phytochemicals can donate hydrogen atoms or electrons to neutralize free radicals, terminate oxidative chain reactions, thereby contributing to the overall radical-scavenging capacity of the extract [4,32,34].

Excessive generation of reactive oxygen species (ROS) can promote oxidative damage to cellular components, including lipids, proteins, and DNA, and have been associated with aging and the pathogenesis of various chronic diseases [4,35]. Therefore, the antioxidant activity observed in the present study provides a plausible biochemical basis for the biological effects of teak leaf extract and supports further investigation of its potential functional food applications.

Anti-inflammatory Activity: The teak leaf extract revealed concentration-dependent inhibition of macrophage migration inhibitory factor (MIF) tautomerase activity, with an IC_{50} value of 10.8 ± 3.1 $\mu\text{g/mL}$ (Figure 2). For comparison, the reference inhibitor ISO-1 exhibited an IC_{50} value of 15.5 ± 0.3 $\mu\text{g/mL}$ [36]. The lower IC_{50} value observed for the teak leaf extract indicates appreciable inhibitory activity against MIF tautomerase. MIF is a pro-inflammatory cytokine involved in regulating innate and adaptive immune responses and has been associated with various inflammatory disorders [37]. Therefore, inhibition of MIF activity has been investigated as a potential approach for modulating excessive inflammatory responses.

The observed inhibitory effect may be associated with the polyphenolic constituents present in teak leaves. Flavonoids and other phenolic compounds have been reported to modulate inflammatory responses through multiple mechanisms, including suppression of pro-inflammatory cytokine production, modulation of the NF- κ B signaling pathway, and attenuation of oxidative stress [4]. Because oxidative stress and inflammation are closely interconnected, the antioxidant activity of teak leaf extract may further contribute to its anti-inflammatory effect. The relatively low IC_{50} value observed in the MIF tautomerase assay suggests that constituents of teak leaves may interact with or inhibit MIF-related molecular pathways. These findings provide preliminary evidence supporting further investigation of

teak leaf phytochemicals as potential sources of bioactive compounds with anti-inflammatory activities.

Antidiabetic Activity: The α -glucosidase inhibitory assay showed that teak leaf extract exhibited inhibitory activity against α -glucosidase, with an IC_{50} value of 86.9 ± 1.1 $\mu\text{g/mL}$ (Figure 3). Although its inhibitory activity was substantially lower than that of the reference inhibitor, acarbose ($IC_{50} = 0.01 \pm 0.00$ $\mu\text{g/mL}$), the extract revealed dose-dependent α -glucosidase inhibition. α -glucosidase is a key digestive enzyme responsible for hydrolyzing complex carbohydrates to glucose, and its inhibition can reduce the rate of carbohydrate digestion and thereby potentially attenuate the postprandial increase in blood glucose levels [38].

The inhibitory activity observed may be associated with the phenolic compounds present in teak leaf extract, which may interact with α -glucosidase, leading to a reduction of catalytic activity. Various plant-derived flavonoids and tannins have been reported to inhibit carbohydrate-hydrolyzing enzymes through different mechanisms [4]. Although the inhibitory potency of the teak leaf extract was lower than that of acarbose, its diverse phytochemical composition may contribute to its overall α -glucosidase inhibitory activity. These findings suggest the potential of teak leaf extract as a natural source of α -glucosidase inhibitory compounds relevant to glycemic management. Nevertheless, further validation through cellular and *in vivo* studies is required.

Tyrosinase Inhibitory Activity: Tyrosinase inhibitory activity is widely used as an *in vitro* indicator of anti-melanogenic potential. The extract exhibited an IC_{50} value of $25,000 \pm 800$ $\mu\text{g/mL}$ (equivalent to 25.0 ± 0.8 g/L) (Figure 4), whereas kojic acid, used as the reference inhibitor, showed a substantially lower IC_{50} value of 88.5 ± 12.1 $\mu\text{g/mL}$. Thus, the tyrosinase inhibitory effect of the teak leaf extract was considerably lower than that of the positive control, suggesting that its potential anti-

melanogenic activity may be limited when evaluated solely based on tyrosinase inhibition.

Tyrosinase is a key enzyme involved in melanin biosynthesis and plays a central role in skin pigmentation and age-related hyperpigmentation. Inhibition of tyrosinase can reduce melanin production and contributes to skin-whitening and antiaging effects [39]. The observed inhibitory activity of teak leaf extract may be associated with its phenolic constituents, which may interact with the enzyme and interfere with its catalytic activity. Furthermore, oxidative stress can influence melanogenesis [40]; therefore, the antioxidant properties of teak leaf extract may potentially contribute to its overall anti-melanogenic potential through mechanisms beyond direct tyrosinase inhibition. However, these possible mechanisms remain to be established experimentally.

Cytotoxicity: The cytotoxicity of teak leaf extract was evaluated using the MTT assay, which assesses cellular metabolic activity based on the reduction of MTT to formazan by metabolically active viable cells. The extract exhibited a concentration-dependent increase in metabolic inhibition (Figure 5). At lower concentrations, only minimal inhibition was observed, indicating limited effects on Vero cell viability. Increasing extract concentrations resulted in greater metabolic inhibition; however, none of the tested concentrations produced 50% inhibition of cell viability. Consequently, the IC_{50} value could not be determined within the tested concentration range, indicating that no detectable cytotoxicity was observed under the experimental conditions.

Formulation Optimization and Confirmation, Proximate and Microbiological Analysis: RSM identified the optimal ratio of mocaf to almond flour by simultaneously considering dietary fiber content, texture, and overall sensory acceptability. The quadratic model exhibited a high adjusted R^2 of 0.9755 and predicted R^2 of 0.9700, indicating a strong agreement between the model and the experimental data and good predictive performance

within the experimental region. In addition, the lack-of-fit was not significant ($p = 0.2582$), further supporting the adequacy of the fitted model for describing the experimental data.

ANOVA further confirmed that the fitted model was highly significant ($p < 0.0001$; $F = 259.88$), indicating that the response was significantly influenced by the flour mixture and its interaction terms. These findings demonstrate that the quality attributes of the cookies were influenced by the relative proportions of mocaf and almond flour, highlighting the importance of optimizing the flour ratio rather than evaluating each ingredient independently.

Multi-response optimization identified a formulation containing 40% mocaf and 60% almond flour as the optimal formulation, with the highest desirability value of 0.985. The model predicted a dietary fiber content of 4.11%, a texture value of 2,846.0 g-force, and an overall sensory acceptability score of 4.0 for this formulation. The high desirability value indicates a favorable overall compromise among the targeted dietary fiber content, textural properties, and sensory acceptability compared with the alternative formulations, including the 60:40 and 50:50 mocaf-to-almond flour ratios.

The optimal formulation identified by RSM, consisting of 40% mocaf and 60% almond flour with 1.5% teak leaf powder, was subsequently evaluated to confirm its sensory performance and overall product quality. The overall sensory acceptability score was classified as category 4, indicating modal overall acceptability response (Table 4). These findings support the suitability of the RSM-derived formulation for producing cookies with acceptable sensory characteristics despite the incorporation of teak leaf powder, which may contribute characteristic color, aroma, and astringency. The proximate composition indicates that the optimized cookies provide a nutritionally relevant matrix for incorporating teak leaf powder as a potential functional

ingredient. Microbiological analysis results indicate a low microbial load and satisfactory microbiological quality of the optimized cookies.

Novelty of the Study, Scientific Innovations, and Practical Implications: This study converts underutilized teak leaf biomass into a value-added functional food ingredient, contributing to sustainable food processing and the utilization of Indonesia's plant biodiversity. The teak leaf extract demonstrated multiple *in vitro* bioactivities, including antioxidant activity, MIF tautomerase inhibition, α -glucosidase inhibition, and tyrosinase inhibition, while showing no detectable cytotoxicity within the tested concentration range. Furthermore, teak leaf powder was successfully incorporated into gluten-free functional cookies formulated with modified cassava flour (mocaf) and almond flour, with RSM used to optimize the formulation. The optimized formulation provided a favorable balance among dietary fiber content, textural properties, and overall sensory acceptability, supporting the potential of teak leaf powder as a functional ingredient in plant-based gluten-free cookies.

The systematic integration of teak leaf extract characterization and cookie formulation in this study directly aligns with key steps of the FFC 17-step framework for functional food development: Product concept and bioactive foundation (phase 1, steps 1–3), Bioactive mechanisms and validation studies (phase 2, step 5), Communication, approval, and market release (phase 3, step 11) [2].

Although processing and incorporation into the cookie matrix may reduce the concentration or activity of some bioactive compounds through dilution and thermal degradation or other processing-related changes [43], previous studies have reported that certain phenolic compounds and antioxidant activities can be retained to varying degrees after baking [41-43]. However, the retention and stability of bioactive compounds in the optimized teak leaf cookies were not directly evaluated

in the present study. Therefore, further investigation is needed to determine the extent to which the bioactive constituents and their activities are preserved following processing. Nevertheless, the optimized cookies demonstrate the feasibility of incorporating teak leaf powder into a gluten-free cookie matrix and provide a promising approach for valorizing teak leaf biomass as a potential functional food ingredient.

CONCLUSION

This study showed that teak leaves represent a promising underutilized plant resource for functional food development, as they contained appreciable levels of phenolic compounds (44.76 ± 1.91 mg GAE/g) and flavonoids (2.16 ± 0.42 mg QE/g), exhibited strong antioxidant activity (DPPH $IC_{50} = 33.5 \pm 2.6$ μ g/mL), MIF tautomerase inhibitory activity ($IC_{50} = 10.8 \pm 3.1$ μ g/mL), and α -glucosidase inhibitory activity ($IC_{50} = 86.9 \pm 1.1$ μ g/mL), and weak tyrosinase inhibitory activity ($IC_{50} = 25.0 \pm 0.8$ g/L). No detectable cytotoxicity was observed within the tested concentration range. Furthermore, RSM successfully optimized the gluten-free cookie formulation containing 1.5% teak leaf powder, identifying a 40:60 mocaf-to-almond flour ratio with a desirability value of 0.985 and favorable dietary fiber content, textural properties, and sensory acceptability. The optimized cookies demonstrated good overall acceptability, low moisture content, a favorable proximate composition, and satisfactory microbiological quality (<10 CFU/g for total plate count and yeast/mold). Overall, these findings support the potential of teak leaf biomass as a sustainable source of bioactive compounds and its practical valorization as a functional food ingredient in gluten-free cookies.

List of Abbreviations: 4-HPP: 4-Hydroxyphenylpyruvate, 4-NPG: 4-Nitrophenyl- α -D-glucopyranoside, CFU: Colony-forming units, DMEM: Dulbecco's modified eagle medium, DMSO: Dimethyl sulfoxide, DPPH: 2,2-Diphenyl-1-picrylhydrazyl, FBS: Fetal bovine serum, GAE: Gallic acid

equivalents, IC₅₀: Half-maximal inhibitory concentration, IL-6: Interleukin-6, MIF: Macrophage migration inhibitory factor, Mocaf: Modified cassava flour, MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PBS: Phosphate-buffered saline, QE: Quercetin equivalents, ROS: Reactive oxygen species, RSM: Response surface methodology, TFC: Total flavonoid content, TNF- α : Tumor necrosis factor-alpha, TPC: Total phenolic content.

Competing Interests: 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.

Authors' Contributions: TK conceptualized and supervised the work, designed the experiments, and critically reviewed the manuscript. ADRD, YP, and PCT critically reviewed the manuscript. EDP and BKIO carried out the experiments and analyses and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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