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ORIGINAL ARTICLE

Exploring the Chemopreventive Potential of Traditional Indonesian Tempeh against Colorectal Cancer: A Metabolomic and Molecular Docking Approach

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ABSTRACT

Colorectal cancer is a leading cause of death globally, characterized by abnormal cellular growths in the colon and rectum. The Leukotriene A4 Hydrolase (LTA4H) enzyme serves as a promising therapeutic target due to its role in driving inflammation and cancer progression. Side effects of conventional therapies prompt the search for alternatives from local functional foods like Tempeh; however, research on the colorectal anticancer potential of various Indonesian non-soy Tempeh variants remains sparse. This study aimed to identify the metabolite profiles of four distinct Indonesian traditional Tempeh types and evaluate their *in-silico* potential as inhibitor candidates against the LTA4H colorectal cancer protein. The examined Tempeh samples included Benguk (Kulonprogo, velvet bean), Menjes Kacang (Malang, peanut), Menjes Gombal (Malang, soy curd residue), and Gembus (Gunungkidul, soy curd residue). Metabolomic profiling was performed utilizing Fourier Transform Infrared (FTIR) spectroscopy and Liquid Chromatography High Resolution Mass Spectrometry (LC-HRMS). Ligand-protein interactions were screened via blind docking using the CB-Dock2 server against the LTA4H target protein (PDB ID: 3U9W). FTIR and LC-HRMS analysis coupled with chemometrics (PCA and PLS-DA) successfully demonstrated clear cluster separations, indicating significant chemical profile variations among the Tempeh types. Out of 22 significant metabolites selected from ANOVA, four distinct compounds representing each Tempeh fulfilled Lipinski's Rule of Five and exhibited the highest binding affinities for LTA4H. These compounds were 1-Linoleoyl glycerol (Benguk; binding affinity −9.3 kcal/mol), daidzein (Menjes Gombal; −8.4 kcal/mol), monoolein (Menjes Kacang; −7.8 kcal/mol), and hexadecanamide (Gembus; −7.7 kcal/mol). These values closely approach or surpass the positive control Bullatalicin (−9.0 kcal/mol), with visualizations verifying stable binding poses within the receptor's active pocket. All four local Tempeh varieties possess unique chemical signatures and hold substantial potential as natural chemopreventive agents against colorectal cancer via LTA4H inhibition, with Benguk (1-Linoleoyl glycerol) demonstrating the strongest inhibitory potential.

1 INTRODUCTION

Colorectal cancer or colon cancer is a malignant growth in the large intestine (colon) and rectum, which is the lowest part of the intestine extending to the anus (Sugeng et al., 2023). Colorectal cancer carries a fairly high mortality risk of approximately 40–50 % (Xi & Xu, 2021). One contributing factor is the uncontrolled production of Leukotriene A4 Hydrolase (LTA4H). Leukotriene A4 Hydrolase (LTA4H) is an enzyme that functions as an epoxide hydrolase and aminopeptidase (Jannah & Sanjaya, 2021). This enzyme plays a role in the carcinogenesis process and converts leukotriene

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A4 into Leukotriene B4, which triggers inflammation and colorectal cancer development. Leukotriene A4 Hydrolase (LTA4H) has been widely used as an inflammatory marker in colorectal cancer, and its inhibition is known to suppress colorectal cancer progression (Mira et al., 2020). The strategic choice of LTA4H as a molecular target stems from its crucial role in orchestrating the inflammatory microenvironment through leukotriene B4 production, directly driving the survival, proliferation, and malignant progression of colorectal cancer cells.

Cancer treatments using chemotherapy, surgery, and radiotherapy cause side effects for patients. One impact of cancer treatment is reduced fertility in childhood and adolescent cancer survivors (Isnaini & Allenidekania, 2024). High toxicity accompanied by minimal efficacy drives the need for alternative colorectal cancer treatments using natural products. Metabolites derived from natural materials generally offer low toxicity, cost, and side effects. Molecular docking serves as a tool to evaluate the potential of natural metabolites as therapeutic candidates for specific targets. Molecular docking is a computational method to predict the binding affinity of a ligand to a receptor protein. Molecular docking is an important aspect of *in silico* therapeutic development (Agu et al., 2023).

Research regarding the potential of natural materials as sources of anticancer metabolites, especially from legume-based functional foods, has been widely conducted. According to Xia et al. (2022), Group B soyasaponins in legumes are known to exert anticancer effects across various colorectal cancer cell lines with DNMT1 and ERK1. In a study by Fitri et al. (2023), glycinin and β-conglycinin peptides from soybean (*Glycine max*) were proven to possess anti-breast cancer activity through *in vitro* and *in silico* tests against the hER-α (3ERT) receptor. Prasetiawati (2023) conducted an *in-silico* study on mung bean (*Vigna radiata*) metabolites, showing that osajin and pomiferin had good affinity against the mutant T790M-L858R-V948R (5HG7) receptor and wild-type (5UGC) lung cancer target receptor, marked by better binding affinity values compared to gefitinib. A molecular docking-based study by Elkhalifa et al. (2023) revealed that genistein in soybeans has therapeutic potential as an anti-breast cancer agent against target proteins 1Y8X (Breast cancer antigen 15.3), 2NVU (Ubiquitin-like protein activation complex), 5T6P (Mucin-1 glycoprotein), and 6CHZ (ER alpha).

The fermentation process of legumes is known to enhance metabolite diversity in the final product. Fermentation of Tempeh can increase both macro and micronutrient content (Fujiana et al., 2021). Fermenting soybeans into Tempeh increases isoflavones in their aglycone forms, such as daidzein, genistein, and glycitein (Nuriyah et al., 2025). Nurkolis et al. (2022) confirmed that Tempeh contains metabolites with anticancer or antitumor effects that play a role in inhibiting cancer cell proliferation, suppressing angiogenesis, and inducing apoptosis in cancer cells. Aside from soybean Tempeh, Indonesia possesses various types of traditional distinctive Tempeh, such as Benguk (raw material of Besengek food from Kulon Progo, made of velvet bean), Menjes Kacang (from Malang, made of peanut), Menjes Gombal (from Malang, made of soybean curd residue), and Gembus (from Gunungkidul, made of soybean curd residue), whose potential as functional foods for colorectal anticancer has not yet been investigated.

Velvet bean (*Mucuna pruriens* L.) contains various bioactive metabolites such as alkaloids, anthraquinones, catechin, and xanthoprotein, with better antioxidant activity compared to other beans like jack bean (*Canavalia ensiformis*) and sword bean. Additionally, velvet bean also exhibits antipyretic effects when tested on male white rats (Putri & Kusuma, 2024). The ethanolic extract of velvet bean seed has anticancer activity against gastric cancer cells by exerting cytotoxic effects, inducing cell death, apoptosis, DNA fragmentation, and cell cycle arrest at the G0/G1 phase (Chinnasamy et al., 2024). Peanuts, as the raw material for Menjes Kacang, are rich in Resveratrol and Vitamin E, which can significantly inhibit the growth and proliferation of HCT-8 colorectal cancer cells through *in vitro* assays (Wang et al., 2021). Meanwhile, Menjes Gombal and Gembus are fundamentally derived from soybeans. However, both are secondary products of soybean (*Glycine max*) processing, namely tofu dregs (soy curd residue).

Based on these reviews, this study focused on identifying colorectal anticancer metabolites in Benguk, Menjes Kacang, Menjes Gombal, and Gembus based on Fourier Transform Infrared Spectroscopy (FTIR), Liquid Chromatography–High Resolution Mass Spectrometry (LC-HRMS), and molecular docking data against the LTA4H target protein of colorectal cancer. We hypothesized that distinct traditional Indonesian Tempeh variants contain unique chemical signatures capable of effectively disrupting colorectal cancer progression by acting as potential inhibitors of the LTA4H enzyme. Therefore, this study systematically integrates FTIR and LC-HRMS for comprehensive metabolomic profiling, chemometrics for clear variance classification, and *in-silico* molecular docking to explicitly screen and validate the specific binding affinities of these local metabolites against the target protein.

2 MATERIALS AND METHODS

2.1 Tempeh Sampling

Tempeh sample selection was evaluated based on physical freshness. Visual criteria for Tempeh included color,

compact texture, distinct aroma, evenly distributed mycelium covering the Tempeh, and maintained cleanliness (Jannah, 2022). Sampling was conducted by directly visiting vendors in traditional markets. Interviews were held with vendors to ensure the fermentation age of the Tempeh was 24 h. The Tempeh samples used are detailed in Table 1.

Table 1. Tempeh samples in this study.

No	Name	Origin Region	Raw Material
1	Menjes Gombal	Malang, East Java	Soybean curd residue (*Glycine max*)
2	Benguk	Kulon Progo, Yogyakarta	Velvet Bean (*Mucuna pruriens*)
3	Menjes Kacang	Malang, East Java	Peanut (*Arachis hypogaea*)
4	Gembus	Gunungkidul, Yogyakarta	Soybean curd residue (*Glycine max*)

2.2 Tempeh Extraction

Tempeh extraction began with flour preparation through drying and grinding processes. Tempeh was cut into cubes, dried in an oven at 50 °C for 24 h, blended, and sieved using an 80-mesh sieve to obtain Tempeh flour. Prior to extraction, the color of the Tempeh flour was tested using a portable chromameter. A total of 50 g of flour was dissolved in 300 mL of methanol for analysis (1:6), homogenized using an ultrasonic bath for 30 min, and filtered with filter paper. The filtrate was evaporated using a rotary evaporator to obtain a thick extract (Alfauzi et al., 2022). It was then left in a fume hood overnight to remove remaining solvent residues, resulting in the extraction yield ready for testing.

2.3 FTIR Spectroscopy Analysis

FTIR spectroscopy analysis was performed using an ATR-FTIR Vertex 70 spectrophotometer (Bruker Optics, Reinstetten, Germany) equipped with an ATR accessory containing a diamond disc crystal as the internal reflection element (Caixeta et al., 2023). Approximately ±2 mg of each fraction was placed on the surface of the ATR crystal to scan the spectrum in the infrared region with a wavenumber range of 4000–400/cm. Spectrum processing and analysis were carried out using Bruker OPUS software (Simatupang et al., 2023). Sample analysis was performed in triplicate.

2.4 Metabolomic Analysis with LC–HRMS

Approximately 5 mg of the extract sample was placed into a 2 mL vial and dissolved in methanol (MS Grade, Merck, Germany). The solution was filtered using a 0.22 µm PTFE syringe filter and injected into the LC-HRMS instrument (Thermo Scientific Q Exactive). Separation was performed on an Accucore™ Phenyl-Hexyl HPLC column (2.6 µm; 2.1 mm diameter × 100 mm length) with a mobile phase consisting of 0.1 % formic acid (Merck, Germany) in water (MS Grade, Merck, Germany) and 0.1 % formic acid in methanol (MS Grade, Merck, Germany). A volume of 250 µL was injected at a flow rate of 0.3 mL/min. Metabolite separation was achieved with a run time of 25 min through positive and negative ESI modes using a gradient method. The mass scanning range was 66.7–1000 m/z. LC-HRMS was utilized to select major metabolites as ligand candidates based on the significance of the metabolites within the Tempeh. Sample analysis was performed in triplicate.

2.5 In Silico Molecular Docking Against Colorectal Cancer Cell Protein

2.5.1 Receptor Preparation

The target protein used was Leukotriene A4 Hydrolase (LTA4H), with its crystal structure obtained from the RCSB Protein Data Bank (PDB code 3U9W) (rcsb.org) (Akash et al., 2024), featuring a resolution of 1.25 Å (Å: Angstrom), and UniProt (uniprot.org) downloaded in .pdb format. The structure file was opened using BIOVIA Discovery Studio Visualizer for purification, which included removing co-crystallized ligands and molecules that could interfere with the ligand docking process. Water molecules were separated from the main structure to purify the PDB protein structure. The structure was then saved in PDB format for the molecular docking stage.

2.5.2 Ligand Preparation

The three-dimensional (3D) structures of the metabolites successfully identified from untargeted LC-HRMS analysis were obtained from the PubChem database in structure-data file (SDF) format (pubchem.ncbi.nlm.nih.gov). Ligand preparation was performed using PyRx, starting with Open Babel, inserting a new item, and importing the 2D or 3D metabolite structures in SDF format. Energy minimization was then conducted ("minimize selected"), and files were saved in Protein Data Bank (PDB) format. The most stable ligand conformation is required to obtain better binding interactions between the ligand and receptor (Hanif et al., 2020). The control ligand used was Bullatalicin, which can act as an LTA4H inhibitor (Noviardi, 2015).

2.5.3 Molecular Docking

Molecular docking was carried out using CB-DOCK2 software with a blind docking approach. The target protein (.pdb) and minimized ligand (.pdb) files were uploaded to the server, and molecular docking was performed. Blind docking was executed on the CB-Dock2 server, utilizing AutoDock Vina with an exhaustiveness value of 8 to ensure an optimized search balance between computational efficiency and conformational space

Table 2. Color testing results of 4 Tempeh types and extraction yields of Benguk, Menjes Kacang, Gembus, and Menjes Gombal.

No	Tempeh Type	Average L^*	Average a^*	Average b^*	Simplicia Weight (g)	Extract Weight (g)	Yield (%)
1	Benguk	69.37	3.77	14.87	50.00	6.410	12.82
2	Menjes Kacang	59.50	7.30	20.70	50.00	12.330	24.67
3	Gembus	73.60	5.97	23.00	50.00	2.267	4.53
4	Menjes Gombal	76.63	5.27	23.67	50.00	5.510	11.02

sampling accuracy. The software automatically identified five potential binding cavities, computing centered search grids around each predicted pocket profile to calculate the binding affinities without predefined search coordinates. The optimal binding area or position was detected automatically. The detected area represents the most stable bond between the ligand and protein. Subsequently, the Vina score (binding affinity) was obtained. The protein-ligand complex file was then saved in .pdb format and opened with Discovery Studio 2021 Client software for molecular docking visualization.

3 RESULTS

3.1 Characterization and Extraction of Tempeh Samples

Benguk Tempeh, made from velvet bean, was characterized by a bright white *Rhizopus* mycelium evenly covering the entire surface, with intact and regularly arranged cream-white velvet bean seeds producing a soft structure. Menjes Kacang, made from peanuts, had a deep brown color with clearly visible peanut granules, which were irregular due to the chopping process prior to fermentation; a thin mycelium covered the peanut surface, resulting in a harder and rougher texture. Menjes Gombal and Tempeh Gembus are both made from tofu dregs, yet they displayed different characteristics. Menjes Gombal was yellowish-white with a surface covered in soft white fuzz, featuring a tight and dense structure. Conversely, Gembus was a pale yellowish-white with a soft, porous, and fragile texture. Visualizations of Menjes Kacang, Benguk, Gembus, and Menjes Gombal can be seen in Figure 1.

The dried Tempeh was subsequently ground and sieved to obtain Tempeh flour. The Tempeh flour from each type can be seen in Figure 2. The drying process was carried out at 50 °C to minimize damage to metabolite content during processing. The Tempeh flour then served as the material for extraction.

Based on the color analysis of the 4 types of Tempeh in Table 2, it was found that the 4 types exhibited color variations, meaning that raw materials significantly influenced the visual color profiles obtained using a portable chromameter across the 4 samples.

3.2 FTIR Spectroscopy Analysis

The FTIR spectra of the 4 types of Tempeh (Benguk, Gembus, Menjes Gombal, and Menjes Kacang) were recorded using FTIR in the wavenumber range of 4000–400/cm, as shown in Figure 3. Functional group interpretation was obtained via IRPal 2.0 software (Ismail et al., 2025).

Spectrum analysis results indicated similarities in several peaks at specific wavenumbers. Furthermore, the FTIR spectrum analysis data of the four Tempeh types demonstrated identical functional groups alongside some differing ones, as presented in Table 3.

FTIR spectrum analysis demonstrated that the four Tempeh types have distinct chemical characteristics, allowing them to be categorized as different types. Benguk and Menjes Kacang exhibited the highest number of unique functional groups, whereas Menjes Gombal and Gembus derived from soybean curd residue (*Glycine max*) shared multiple similarities, yet still displayed their own characteristic functional groups. Generally, all four Tempeh types were rich in C–O groups (carbohydrates, esters, lipids), C–H groups (lipids, fatty acids), and Ar–CH=CHR groups (proteins, isoflavones).

The PCA score plot using PCs to differentiate the four Tempeh samples is shown in Figure 4A. The horizontal axis (PC 1) explains the main raw material factor by 57 %, and the vertical axis (PC 2) explains environmental factors during fermentation by 31.8 %, with a total variability of 88.8 %. Each sample type is shown in a different color: Benguk (pink), Gembus (green), Menjes Gombal (dark blue), and Menjes Kacang (light blue) lie in clearly separated clusters within the PCA space, indicating that each Tempeh type possesses significantly different characteristics. Although Gembus and Menjes Gombal share the same raw material, their clusters were far apart. This was influenced by differences in environmental factors during the fermentation process. In addition, Menjes Gombal is processed through further fermentation, sometimes with a slight addition of soy residue (Anisa et al., 2021). In other literature, Menjes Gombal is made entirely from soy residue (Hamida & Purnomo, 2024).

Variable Importance in Projection (VIP) was used in PLS-DA to assess the relative importance of each variable in differentiating sample groups, where VIP scores $\geq$ 1 are generally considered the standard for

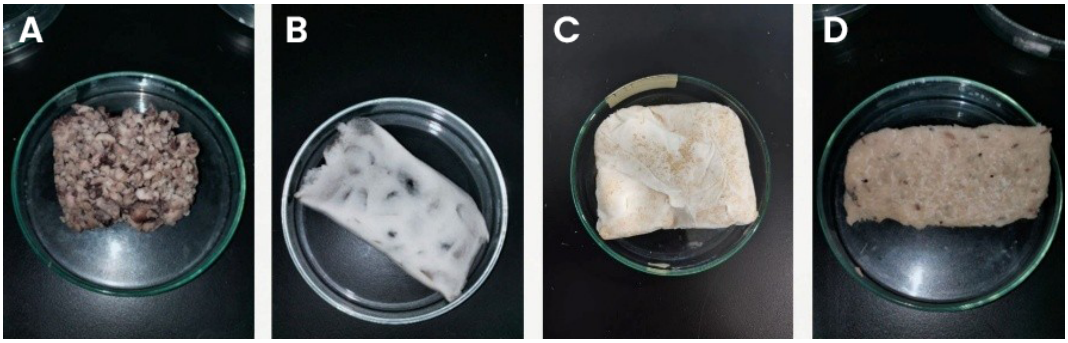


Figure 1. Characterization of Tempeh Samples. (A) Menjes Kacang Tempeh, (B) Benguk Tempeh, (C) Gembus Tempeh, (D) Menjes Gombal Tempeh.

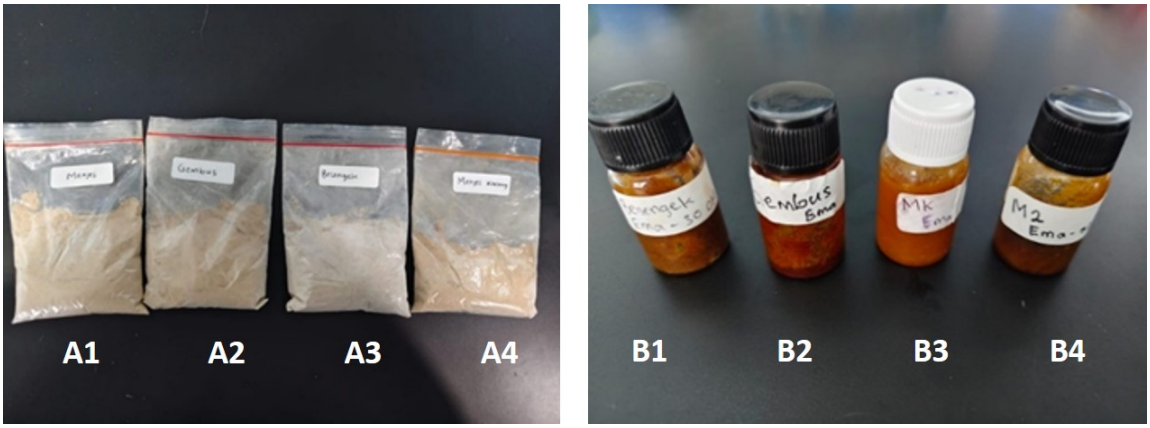


Figure 2. Comparison of tempeh variants. Left: Tempeh flours of Menjes Gombal (A1), Gembus (A2), Benguk (A3), and Menjes Kacang (A4). Right: Corresponding extracts of Benguk (B1), Gembus (B2), Menjes Kacang (B3), and Menjes Gombal (B4).

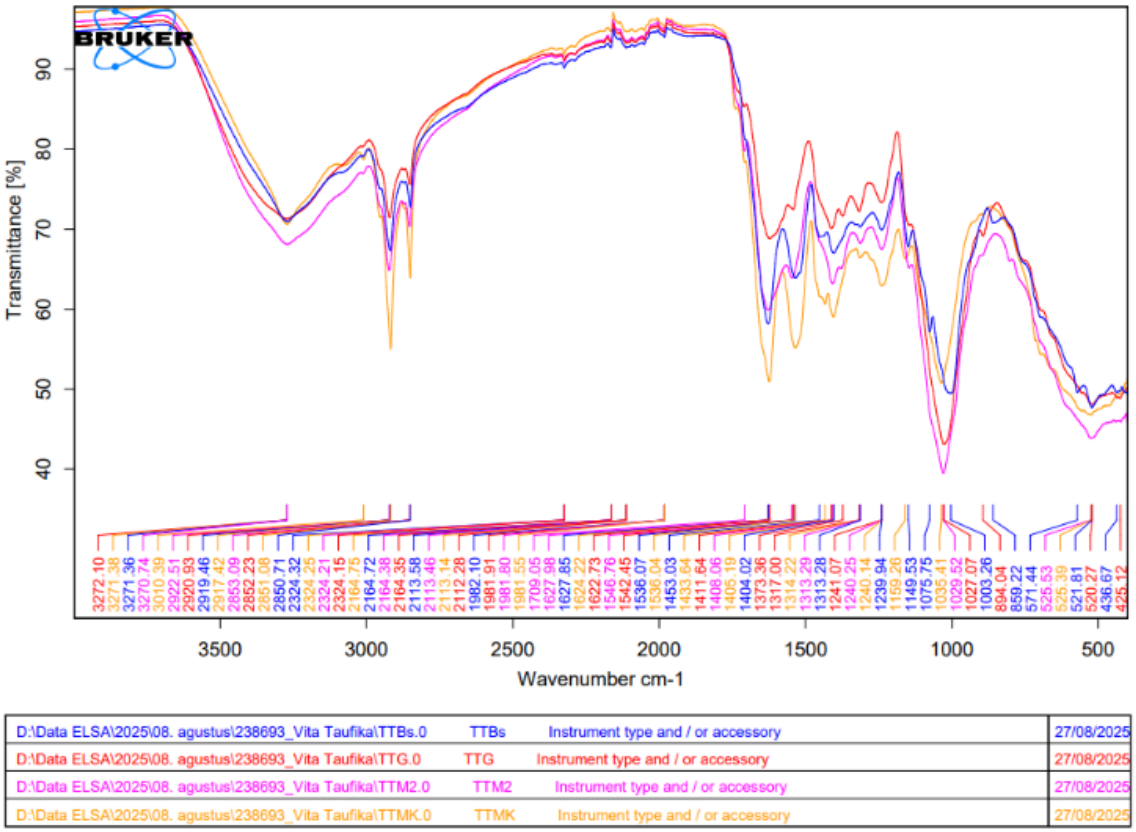


Figure 3. FTIR transmittance spectrum of 4 Tempeh types.

Table 3. FTIR spectrum analysis data of 4 Tempeh types (similarities and differences).

No	Wavenumber (1/cm)				Area (1/cm)	Class	Functional Group
	Benguk	Gembus	Menjes Gombal	Menjes Kacang			
1	3271.36	3272.10	3270.74	3271.38	3270–3330	Alkynes	≡C–H stretch
2	–	–	–	3010.39	3000–3100	Aromatics	Ar–H stretch
3	2919.46	2920.93	2922.51	2917.42	2850–3000	Alkanes	C–H stretch
4	2850.71	2853.23	2853.09	2851.08	2845–2855	Alkanes	$-CH_2-$
5	2324.32	2324.15	2324.21	2324.25	2280–2440	Misc.	P–H phosphine sharp
6	2164.72	2164.35	2164.38	2164.75	2110–2165	Misc.	Isocyanides
7	2113.58	2112.28	2113.46	2113.14	2110–2140	Alkynes	C≡C stretch
8	1982.10	1981.91	1981.80	1981.55	1980–2140	Misc.	N=C in R–N=C=S
9	–	–	1709.05	–	1705–1715	Carboxylic acids	Dimer C=O
10	1627.85	1622.73	1627.98	1624.22	1620–1630	Alkenes	Ar–CH=CHR
11	–	1542.45	1546.76	–	1530–1550	Amides	N–H out of plane
12	1536.07	–	–	1536.04	1535–1545	Misc.	Alif. Nitro
13	1453.03	–	–	–	1450–1470	Alkenes	CH_2 and CH_3
14	–	–	–	1433.64	1400–1500	Aromatics	Ar C–C stretch
15	1404.02	1411.64	1408.06	1405.19	1400–1500	Aromatics	Ar C–C stretch
16	–	1373.36	–	–	1370–1380	Alkanes	CH_2 and CH_3
17	1313.28	1317.00	1313.29	1314.22	1300–1350	Misc.	S=O sulfone 1
18	1239.94	1241.07	1240.25	1240.14	1200–1300	Misc.	N–O aromatic
19	1149.53	–	–	1159.26	1120–1160	Misc.	S=O sulfone 2
20	1075.75	–	–	–	1070–1150	Ethers	C–O stretch
21	–	1027.07	1029.52	1035.41	1000–1110	Misc.	Si–OR (broad)
22	1003.26	–	–	–	1000–1320	Carboxylic acids	C–O stretch
23	–	894.04	–	–	885–895	Alkenes	=CH out of plane
24	859.22	–	–	–	855–870	Aromatics	C–H out of plane
25	571.44	–	–	–	500–680	Alkyl halides	C–Br stretch
26	521.81	520.27	525.53	525.39	500–680	Alkyl halides	C–Br stretch
27	436.67	–	–	–	400–500	Alkyl halides	C–Br stretch
28	–	425.12	–	–	400–500	Alkyl halides	C–Br stretch

distinguishing variables. Figure 4B shows that 25 distinguishing wavenumbers with a VIP score ≥ 1 were obtained. The wavenumber with the highest VIP score was 2916.060 51/cm, with an average VIP score of 2.20. The sequence of abundance for this wavenumber from highest to lowest was Gembus, Benguk (Besengek), Menjes Gombal (Menjes), and Menjes Kacang. The wavenumber range of 2800–3300/cm depicts the C–H functional group. The absorption of the C–H functional group appeared at wavenumber 2916/cm (Istinanda et al., 2019). The wavenumber 2916.060 51/cm belongs to the methylene group ($-CH_2-$) (Fan et al., 2025). This methylene group is categorized within lipids or methyl lipids (Delikatny et al., 2011).

3.3 Metabolomic Analysis with LC–HRMS

The PCA score plot analysis of the LC-HRMS data from the 4 Tempeh types in Figure 5(A) shows that the components contained in the four Tempeh types possessed good variance, with clear cluster separation for each Tempeh. This can be observed from the X-axis (PC 1) total: 30 %, describing differences in major metabolites related to substrate and nutrient composition. The Y-axis (PC 2): 22.7 %, describes environmental differences during the fermentation process. The total variance explained by these two components was 52.7 %.

Figure 5(B) displays a heatmap representing variations in the relative abundance of metabolites across the 4 Tempeh types. The metabolite content of Menjes Kacang included (±)12,13-DiHOME, 13-HODE, Kojic acid, Monoolein, Propionylcarnitine, Sphingosine, Oleoyl ethanolamide, N-Acetylhistamine, Nicotinic acid, and Ethyl dodecanoate. Benguk Tempeh (Besengek) showed high intensity in Citric acid, Ethyl myristate, Ethyl oleate, Pyridoxine, Nervonic acid, 1-Linoleoyl glycerol, Aspartylphenylalanine, and L-Methionine sulfoxide. Gembus was dominant in Valylproline, Hexadecanamide, L-(+)-Arginine, and Ethyl dodecanoate. Menjes Gombal (Menjes) had high expression toward Methyl palmitate, Glutamic acid, Histidine, Daidzein, L-Methionine sulfoxide, and L-(+)-Arginine. Figure 5(C) shows the VIP scores of LC-HRMS for the four Tempeh types, providing a visualization of distinguishing metabolite distributions in Benguk Tempeh (Besengek), Menjes Kacang, Menjes Gombal (Menjes), and Gembus, which are listed in Table 4.

The metabolites used for *in silico* molecular docking testing were the significant metabolites identified by

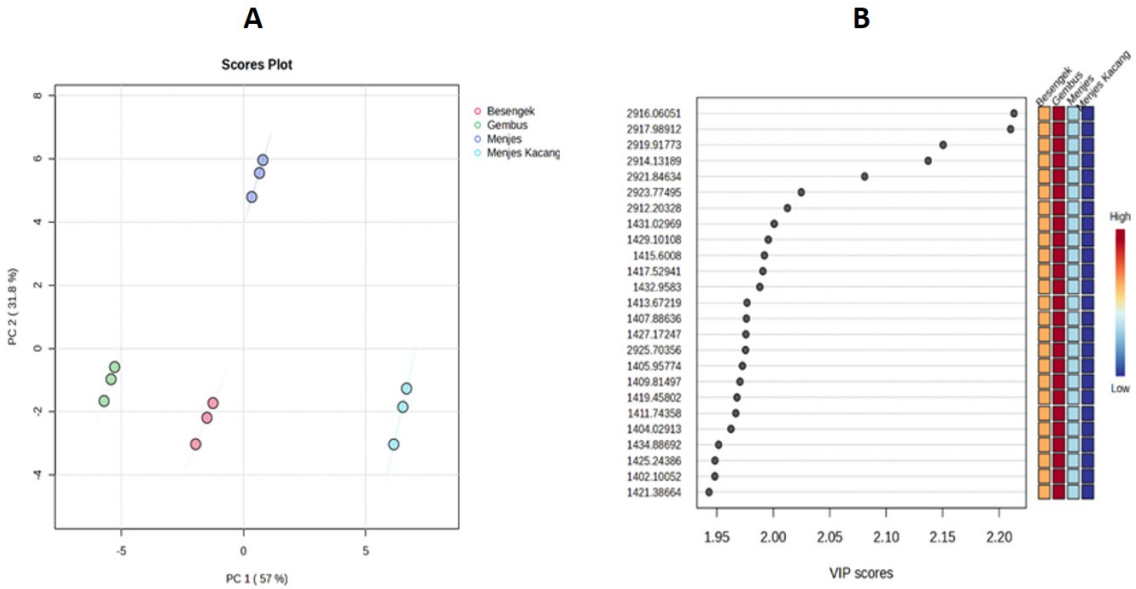


Figure 4. (A) Principal Component Analysis (PCA) score plot using Principal Component (PC1) and (PC2) of four Tempeh types. (B) VIP Scores from PLS-DA FTIR of four Tempeh types. Besengek (Benguk Tempeh, Kulonprogo), Gembus (Gunungkidul), Menjes (Menjes Gombal, Malang), and Menjes Kacang (Kacang Tempeh, Malang).

Table 4. Significant metabolites based on ANOVA via MetaboAnalyst 6.0.

No	Metabolite	Molecular Formula	Molecular Weight	RT (min)	Area (max)
1	(±)12(13)-DiHOME	$C_{18}H_{34}O_4$	314.245 44	11.176	1,386,384,373
2	(±)13-HODE	$C_{18}H_{32}O_3$	296.235 07	12.554	832,399,077
3	Nervonic acid	$C_{24}H_{46}O_2$	366.349 63	16.194	26,138,402
4	1-Linoleoyl glycerol	$C_{21}H_{38}O_4$	354.276 49	14.186	6,190,595,160
5	L-(+)-Arginine	$C_6H_{14}N_4O_2$	174.111 72	0.810	270,990,207
6	Hexadecanamide	$C_{16}H_{33}NO$	255.256 01	14.407	5,617,480,045
7	Glutamic acid	$C_5H_9NO_4$	147.053 12	0.912	934,528,184
8	Sphingosine	$C_{18}H_{37}NO_2$	299.281 88	11.403	28,547,878
9	Histidine	$C_6H_9N_3O_2$	155.069 48	0.851	662,683,437
10	Ethyl Dodecanoate	$C_{14}H_{28}O_2$	228.208 76	15.510	62,029,246
11	Nicotinic acid	$C_6H_5NO_2$	123.032 04	1.025	1,921,697,339
12	Citric acid	$C_6H_8O_7$	192.026 34	1.019	242,650,526
13	Kojic acid	$C_6H_6O_4$	142.026 73	1.168	50,617,905
14	Daidzein	$C_{15}H_{10}O_4$	254.057 87	7.297	1,920,762,746
15	Methyl palmitate	$C_{17}H_{34}O_2$	270.255 79	17.071	932,475,353
16	Monoolein	$C_{21}H_{40}O_4$	356.292 18	14.821	8,805,308,272
17	N-Acetylhistamine	$C_7H_{11}N_3O$	153.090 24	0.868	79,117,703
18	Propionylcarnitine	$C_{10}H_{19}NO_4$	217.131 45	1.166	36,244,278
19	Pyridoxine	$C_8H_{11}NO_3$	169.073 88	0.972	60,731,292
20	Valylproline	$C_{10}H_{18}N_2O_3$	214.131 77	1.284	299,766,648
21	Aspartylphenylalanine	$C_{13}H_{16}N_2O_5$	280.106 16	2.438	31,573,969
22	Ethyl oleate	$C_{20}H_{38}O_2$	310.286 80	17.771	924,346,580

ANOVA on MetaboAnalyst 6.0. Significant metabolites were used because they are not only statistically significant but also biologically critical in separating sample groups. The choice of ANOVA data (significant metabolites) was grounded on statistical selection. A total of 22 significant metabolites were obtained, as shown in Table 4.

3.4 In Silico Molecular Docking Against LTA4H Colorectal Cancer Protein

A total of 22 significant metabolites underwent molecular docking using CB-Dock2 to determine their binding affinity values. Selection was then conducted based on these values. Visualization was carried out on metabolites displaying the lowest binding affinities. A lower binding affinity indicates a stronger potential binding activity with the target receptor. Furthermore,

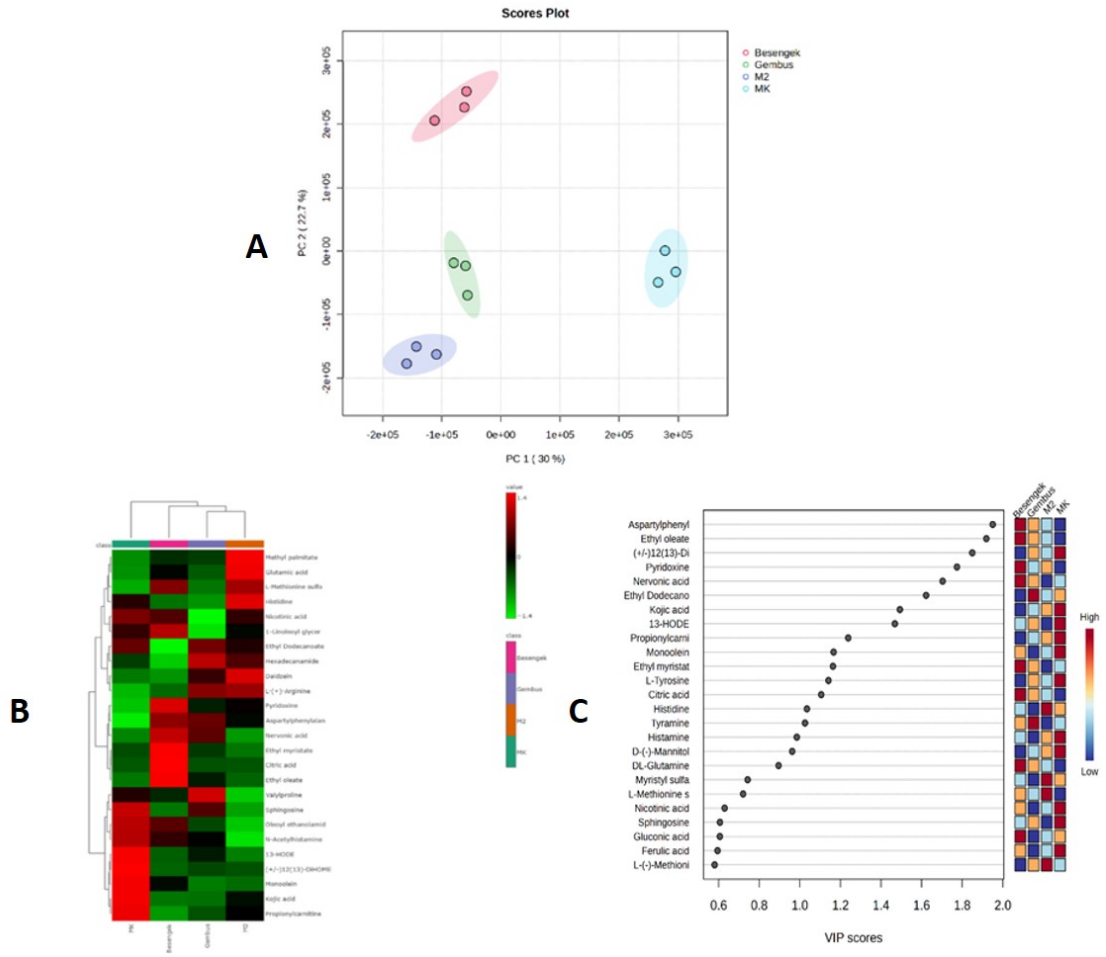


Figure 5. Multivariate analysis of 25 metabolites from 4 Tempeh types. (A) Principal Component Analysis (PCA) scores plot of LC-HRMS data from 4 Tempeh types. (B) LC-HRMS heatmap representing variation in relative metabolite abundance across 4 Tempeh types. (C) Variable Importance in Projection (VIP) score of LC-HRMS for the 4 Tempeh types. Besengek (Benguk Tempe, Kulonprogo), Gembus (Gunungkidul), Menjes (Menjes Gombal, Malang), and Menjes Kacang (Kacang Tempeh, Malang).

Table 5. Druglikeness prediction of selected metabolites.

Parameter	1-Linoleoyl glycerol	Daidzein	Monoolein	Hexadecanamide
Formula	$C_{21}H_{38}O_4$	$C_{15}H_{10}O_4$	$C_{21}H_{40}O_4$	$C_{16}H_{33}NO$
Molecular Weight (g/mol)	354.52	254.24	356.54	255.44
HBA	4	4	4	1
HBD	2	2	2	1
Log *P*	0.94	2.24	5.09	4.83
Lipinski	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation

drug-likeness predictions of selected metabolites were performed (Table 5), revealing that 1-Linoleoyl glycerol, daidzein, monoolein, and hexadecanamide complied with the Lipinski Rule of Five criteria in SwissADME. This assessment is vital to determine whether a metabolite is safe for oral drug consideration.

Table 6 displays the molecular docking results of the four metabolites with the lowest binding affinities against the target LTA4H protein (PDB ID 3U9W), along with their matching hydrogen bonds. Hydrogen bonds represent the most stable and strong interactions in molecular docking.

In addition to binding affinity, amino acid residues within the active site serve as another key parameter in docking visualization. The 3D docking visualizations of Bullatalicin (control), 1-Linoleoyl glycerol, Hexadecanamide, Daidzein, and Monoolein in Figures 6–9 (A, B, C) consistently showed ligand poses demonstrating that the metabolites fit inside the protein structure, specifically positioned between helices and loops. This indicates that the ligands are relatively stable within the pocket.

Figures 6–9 (D) show the 2D amino acid residue interaction visualizations of the ligands Bullatalicin

Table 6. Molecular docking results of metabolites with the lowest binding affinities against target protein LTA4H (PDB ID 3U9W).

No.	Metabolite	Molecular Formula	Binding Affinity (kcal/mol)	Hydrogen Bonds
1	Bullatalicin (control)	$C_{37}H_{66}O_8$	−9.0	GLY1268; ARG1563; GLN1136; GLY1269
2	1-Linoleoyl glycerol	$C_{21}H_{38}O_4$	−9.3	TYR1267; TYR1378; SER1379; PHE1362
3	Daidzein	$C_{15}H_{10}O_4$	−8.4	ARG1563; TYR1378
4	Monoolein	$C_{21}H_{40}O_4$	−7.8	LYS1364; ASP1312
5	Hexadecanamide	$C_{16}H_{33}NO$	−7.7	GLN1136; GLU1318; HIS1299; TYR1383

(control), 1-Linoleoyl glycerol, Hexadecanamide, Daidzein, and Monoolein with the LTA4H target protein. All five metabolites displayed close interactions with key residues. Daidzein shared multiple amino acid residues with the Bullatalicin control, specifically at ARG1563, TYR1378, LYS1565, HIS1295, and MET1564. Monoolein shared many bond similarities with the bullatalicin control, including alkyl bonds with MET1270, PRO1374, ALA1137, and π-alkyl bonds with TRP1311, TYR1378, HIS1295, PHE1314. Monoolein was categorized as a distinguishing metabolite with a low binding affinity value of −7.8 kcal/mol. However, monoolein only shared alkyl and π-alkyl bonds with the bullatalicin control, lacking shared hydrogen bonds with the control metabolite. Hexadecanamide yielded the highest binding affinity value among the selected metabolites (−7.7 kcal/mol). Nevertheless, hexadecanamide shared numerous bonds with bullatalicin control, including a conventional hydrogen bond at GLN1136. Furthermore, it shared alkyl bonds with PRO1374, ALA1377, ALA1137, MET1270, and π-alkyl bonds with TRP1311, TYR1267, TYR1378, PHE1314. 1-Linoleoyl glycerol shared amino acid residues with the Bullatalicin control at the conventional hydrogen bond TYR1267, carbon-hydrogen bond TYR1378, alkyl bonds MET1270, ALA1377, LEU1369, and π-alkyl bonds PHE1314, TRP1311.

Overall, all four metabolites possess potential as colorectal anticancer candidates. This is supported by their shared amino acid residue bonds with the control metabolite Bullatalicin and binding affinity values that approach or are lower than bullatalicin (−9.0 kcal/mol). Monoolein is abundantly found in Menjes Kacang. Monoolein lies within the wavenumber range of 1045–1261/cm, representing the C−O functional group (Nehru et al., 2025). 1-Linoleoyl glycerol is highly contained in Benguk. 1-Linoleoyl glycerol, also known as 1-monolinolein, is categorized within lipid derivatives (Shuai et al., 2021). 1-Linoleoyl glycerol falls under the monoacylglycerol class. This monoacylglycerol is present at a weak wavenumber around 3008/cm, assigned to the C−H stretching mode, ν(C−H), and the cis-disubstituted double bond. Asymmetric and symmetric C−H stretching from the methylene groups of the fatty acid chain can be observed between wavenumbers 2922 and 2853/cm. This wavenumber also indicates a high CH_2/CH_3 ratio (Hobuss et al., 2020). Daidzein is highly contained in Menjes Gombal. Soybean-derived food products such as Tempeh contain daidzein (Singh et al., 2023). Based on a study by Sulistyowati et al. (2020), the FTIR spectrum of soybean powder containing daidzein showed wavenumbers 2925/cm and 2855/cm originating from CH_3 and CH_2 groups in stretching mode. This aligns with the FTIR findings of this study, where wavenumbers 2850–3000/cm fall under the C−H group and wavenumbers 2845–2855/cm belong to the CH_2 group. Hexadecanamide is highly contained in Gembus. No literature has yet evaluated hexadecanamide in legumes and its anticancer potential. However, hexadecanamide satisfies oral drug standards based on the Lipinski Rule of Five (Muslikh et al., 2025).

4 DISCUSSION

The evaporation process yielded thick extracts of the four Tempeh types (Figure 2). Extraction of the four Tempeh types in Table 2 was conducted using identical weights of Tempeh flour, which was 50 g. Menjes Kacang and Benguk achieved the highest yield values, at 24.67 % and 12.82 %, respectively. Meanwhile, Gembus had the lowest yield at 4.53 %, and Menjes Gombal stood at 11.02 %. The yield difference between Menjes Gombal and Gembus, which share the same raw material (soybean tofu dregs), reflects differences in their physical characteristics. Gembus is porous/hollow, whereas Menjes Gombal possesses a dense texture. Tempeh texture formation is influenced by moisture content, fat content, and Tempeh type (Widiastuti et al., 2024). Variations in yield values are influenced by several factors such as simplicia size, solvent type, and an increase in solvent concentration can also enhance yield (Kusuma & Aprileili, 2022). Other factors include the temperature method, particle size, and the sample-to-solvent ratio (Wadli & Hasdar, 2021). Raw material composition also affects extraction outcomes (Efendi et al., 2020). Extraction yield represents the ratio between the dry weight of the extract product and the initial raw material weight. A higher yield value indicates a larger content of bioactive components within the sample (Senduk et al., 2020). Methanol is a universal solvent, meaning it can dissolve most metabolites that are polar and non-polar. Methanol solvent can yield excellent extraction of secondary metabolites (Adisti et al., 2023).

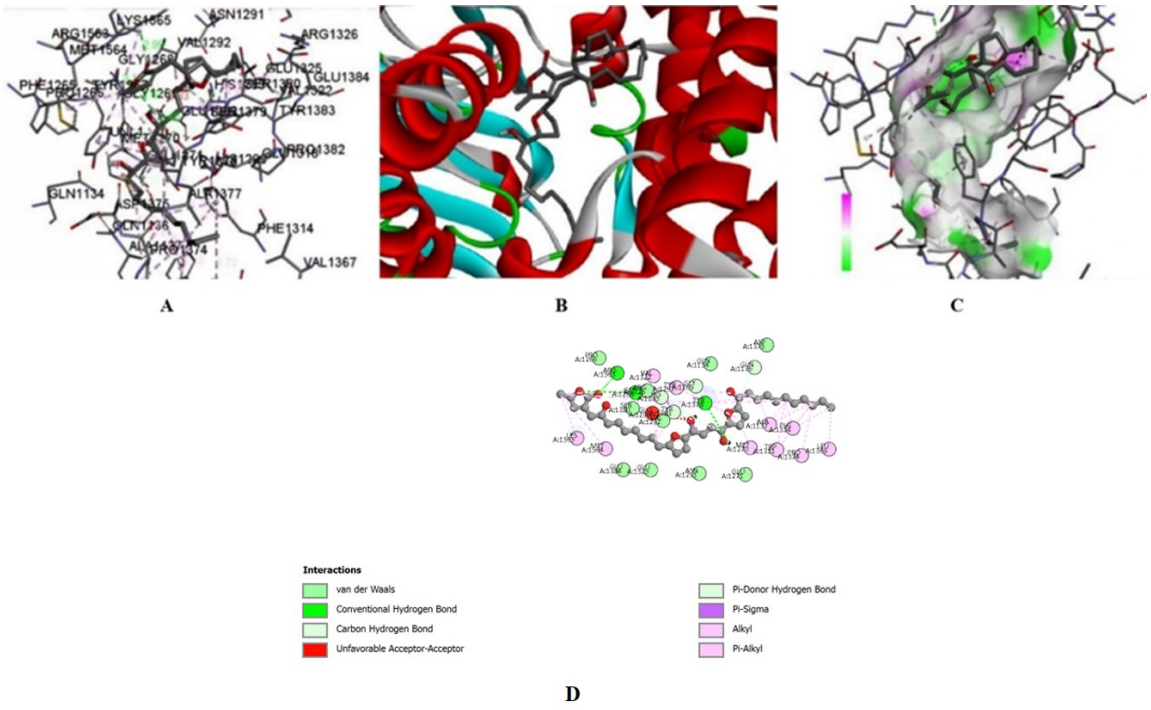


Figure 6. 3D molecular docking visualization: (A) Amino acid residues, (B) Ligand pose, (C) Binding pocket, and 2D (D) amino acid residues of the control ligand Bullatalicin with LTA4H target protein.

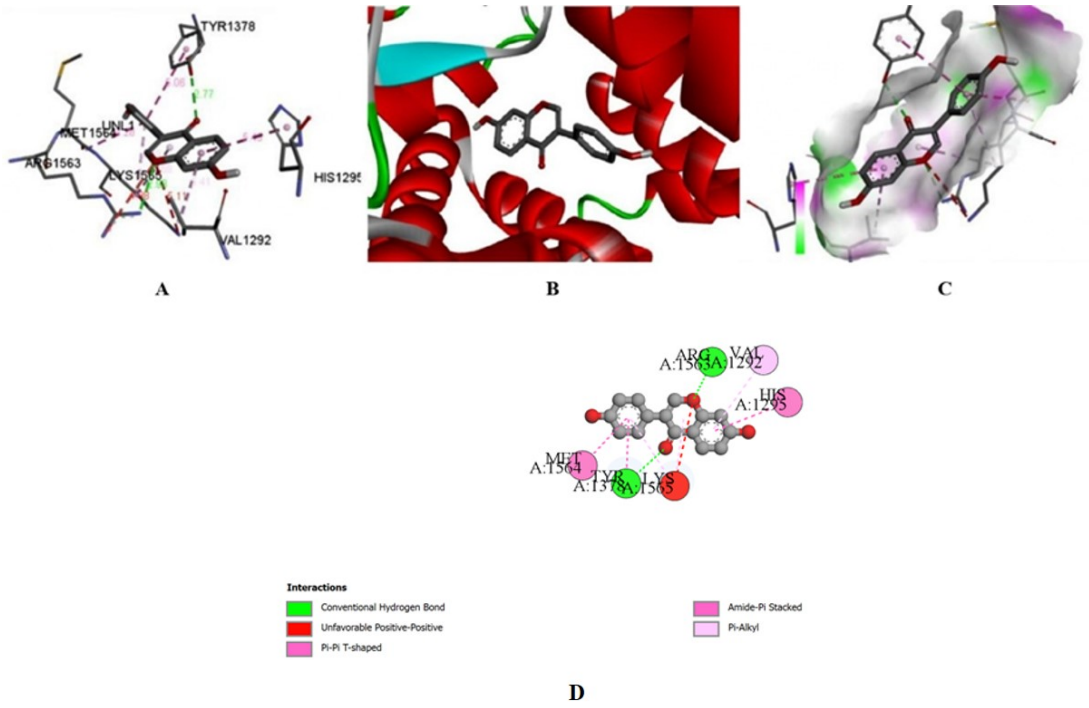


Figure 7. 3D molecular docking visualization: (A) Amino acid residues, (B) Ligand pose, (C) Binding pocket, and 2D (D) amino acid residues of Daidzein ligand with LTA4H target protein.

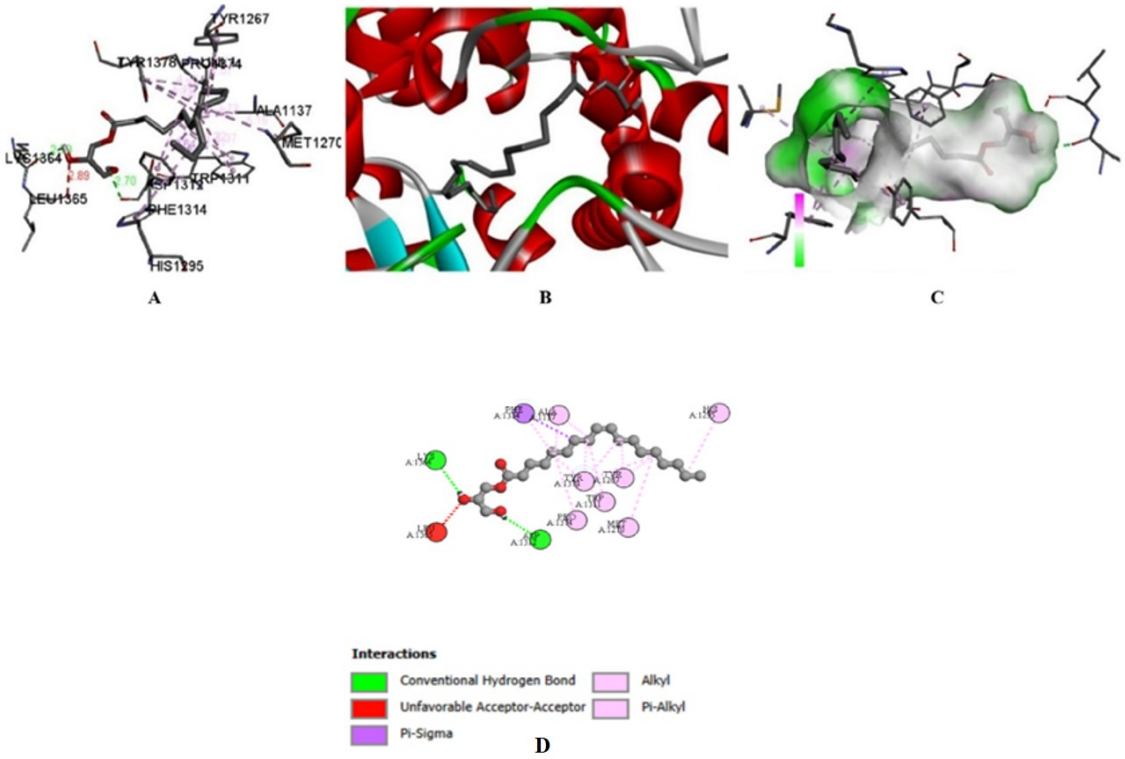


Figure 8. 3D molecular docking visualization: (A) Amino acid residues, (B) Ligand pose, (C) Binding pocket, and 2D (D) amino acid residues of Monoolein ligand with LTA4H target protein.

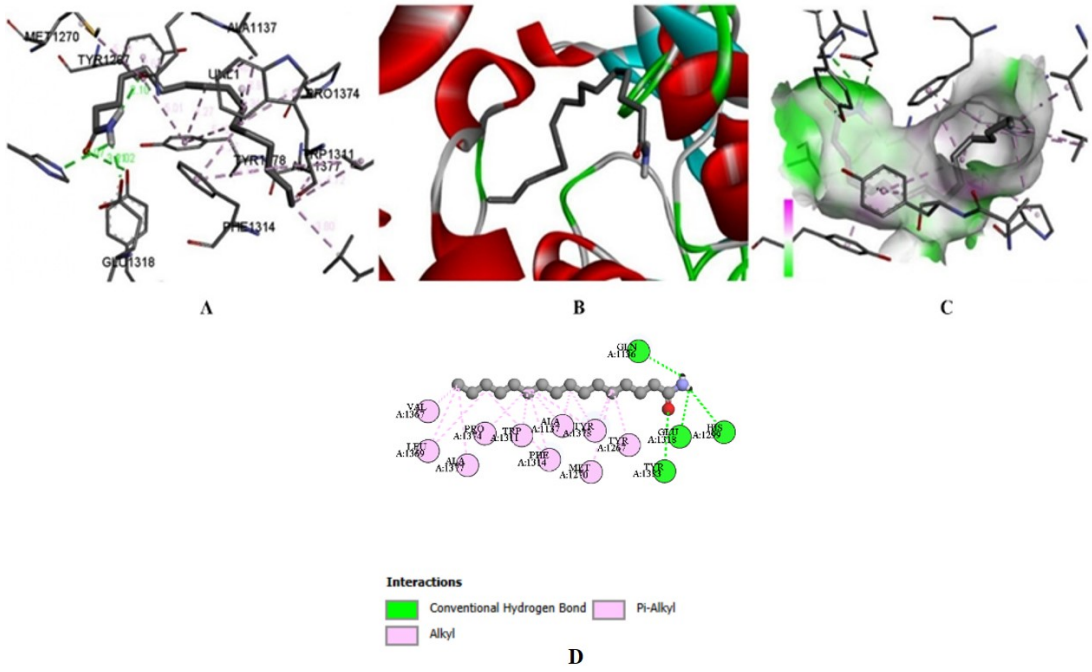


Figure 9. 3D molecular docking visualization: (A) Amino acid residues, (B) Ligand pose, (C) Binding pocket, and 2D (D) amino acid residues of Hexadecanamide ligand with LTA4H target protein.

Based on PCA score plots, the clear cluster separation (Figure 4)—distinguishing tofu dregs (Gembus and Menjes Gombal) from those derived from peanuts (Menjes Kacang) and velvet beans (Benguk)—is driven by differences in the base substrate and the metabolic activity of the *Rhizopus* mold (Ginting et al., 2024). The identification of the wavenumber 2916.06/cm as a primary discriminator—with the highest Variable Importance in Projection (VIP) score (2.20)—reflects the asymmetric stretching vibration of methylene ($-CH_2-$) groups, which are inherently abundant in lipid structures. This aligns with previous studies indicating that shifts or intensity variations in the 2925–2850/cm region are closely linked to the hydrolysis of triglycerides into free fatty acids by fungal lipase enzymes during solid-state fermentation (Zhang et al., 2022). Furthermore, the presence of other functional groups—such as amides (C=O stretching and N–H bending at 1627–1542/cm) and aromatic C–C bond signals in the fingerprint region—indicates an abundance of bioactive peptides and glycosylated isoflavones that remain well-preserved within the methanol extracts.

The high abundance of 1-linoleoyl glycerol (a monoacylglycerol) in Benguk demonstrates the enzymatic efficiency of *Rhizopus* in converting complex lipids derived from *Mucuna pruriens* seeds. Meanwhile, the high expression of daidzein in Menjes Gombal confirms that biotransformation occurs during fermentation, wherein the fungal enzyme β-glucosidase hydrolyzes glycosidic bonds in the soybean substrate, yielding a far more bioactive aglycone form (Nurmillah et al., 2025). The detection of monoolein in Menjes Kacang and hexadecanamide in Gembus underscores that food processing residues (such as tofu dregs or legume by-products) retain significant functional potential, unlocked through the secondary metabolic modifications driven by the fungi and bacteria (Wu et al., 2022). This diversity of compounds confirms that the use of local, non-soybean raw materials can induce novel, protective metabolite profiles not present in standard soybean-based Tempeh.

The highest binding affinity was observed for the compound 1-linoleoyl glycerol (−9.3 kcal/mol), indicating a more spontaneous and stronger thermodynamic interaction compared to the positive control, bullatalicin (−9.0 kcal/mol) (Table 6). This stability is facilitated by the formation of a robust network of conventional hydrogen bonds with the key residue TYR1267, as well as hydrophobic (π-alkyl) interactions within the pocket formed by the aromatic amino acids TRP1311 and PHE1314. Although the ligands daidzein (−8.4 kcal/mol), monoolein (−7.8 kcal/mol), and hexadecanamide (−7.7 kcal/mol) exhibit free energy values slightly higher than that of the control, 3D pose visualization confirms that all these compounds fit within the receptor's active cavity, situated among its helical folds. Structurally blocking this active site theoretically prevents the enzyme from converting leukotriene A4 into the pro-inflammatory mediator leukotriene B4 (LTB4) (Chen et al., 2004)—a process known to be a primary driver of the tumor microenvironment.

Collectively, the integration of macro- and micro-chemical approaches successfully substantiates the therapeutic potential of traditional, non-soy-based Indonesian tempeh as a natural chemopreventive agent against colorectal cancer. An assessment of "drug-likeness" reveals that the four primary metabolites—1-linoleoyl glycerol, daidzein, monoolein, and hexadecanamide—adhere perfectly to Lipinski's Rule of Five with zero violations, confirming excellent oral bioavailability and intestinal absorption. This is further supported by recent literature, which indicates that the lipid compound 1-linoleoyl glycerol and the isoflavone daidzein exhibit significant cytotoxic bioactivity capable of inducing apoptosis in cancer cells and suppressing the release of inflammatory chemokines (Chatterjee et al., 2026; Han et al., 2015). Consequently, molecular docking data for LTA4H—which correlates linearly with chemical fingerprinting derived from FTIR and HRMS analysis—demonstrate that local tempeh variants, particularly Benguk Tempeh, possess strong, computationally-derived clinical relevance, warranting further development as an anti-inflammatory and anti-colorectal cancer dietary preventive measure.

5 CONCLUSIONS

FTIR and LC-HRMS metabolomic profiling coupled with chemometrics successfully differentiated the chemical footprints of four Indonesian local Tempeh varieties. Benguk Tempeh (raw material of Besengek food, velvet bean) and Menjes Kacang (peanut) displayed the highest functional group variations, while Menjes Gombal and Gembus exhibited similar baseline spectra from shared soy curd residue, but maintained distinct environmental cluster separations. Furthermore, *in silico* molecular docking against the LTA4H colorectal cancer target protein (PDB ID: 3U9W) screened four primary metabolite biomarkers fulfilling Lipinski's drug-likeness rules. They established highly stable binding networks via key hydrogen and hydrophobic bonds inside the protein's active site pocket. The calculated binding affinities include: 1-Linoleoyl glycerol (Benguk; −9.3 kcal/mol), daidzein (Menjes Gombal; −8.4 kcal/mol), monoolein (Menjes Kacang; −7.8 kcal/mol), and hexadecanamide (Gembus; −7.7 kcal/mol). These values closely match or exceed the standard positive control Bullatalicin (−9.0 kcal/mol). Overall, these results demonstrate that local traditional Tempeh variants possess significant

functional potential as natural chemopreventive sources against colorectal cancer via structural LTA4H enzyme inhibition, with Benguk (1-Linoleoyl glycerol) offering the strongest computer-aided therapeutic candidate.

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Fitri Setya Amanda: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft. **Ema Damayanti:** Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Project administration, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing. **Tsania Taskia Nabila:** Investigation, Formal analysis, Visualization, Writing – review & editing. **Ismail Ismail:** Formal analysis, Validation, Writing – review & editing. **Vita Taufika Rosyida:** Resources, Writing – review & editing. **Siska Aditya:** Resources, Validation, Writing – original draft, Writing – review & editing. **Wirawan Adikusuma:** Formal analysis, Validation, Writing – review & editing. **Artini Pangastuti:** Conceptualization, Methodology, Supervision, Writing – review & editing.

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DECLARATION OF 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.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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