CHEM PUBLISH
Chempublish Journal
Journal Homepage: online-journal.unja.ac.id/chp
Department of Chemistry
Faculty of Science and Technology
Universitas Jambi
ISSN (Online): ISSN 2503-4588
No. 23/E/KPT/2019

Article

Comparative Study of Macerated and Soxhlet-Extracted *Moringa oleifera* Leaf Extracts: LC-MS-Based Metabolomic Profiling, Antioxidant Activity, and In Silico Target Prediction

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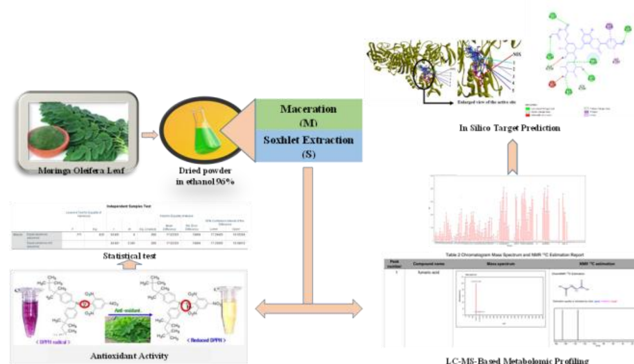
Abstract

Moringa leaves (*Moringa oleifera* L.) are rich in secondary metabolites such as flavonoids, alkaloids, tannins, saponins, and terpenoids, which function as natural antioxidants. This study aimed to analyze the metabolite profile of M. oleifera leaf extracts obtained through two extraction techniques using LC-MS, evaluate their antioxidant activity via the DPPH assay, and predict the interaction between NADPH oxidase (as a receptor) and key plant-derived compounds through molecular docking. LC-MS results indicated that the maceration method yielded 101 secondary metabolites, with flavonoid derivatives comprising 70.99% of the extract, dominated by five key compounds including Kaempferol 3-O-robinobioside and Luteolin-7-glucoside. In contrast, the Soxhlet method resulted in 83 identified compounds, with a higher proportion of flavonoids (75.61%), and prominent compounds including quercetin-3-O-glucoside and Kaempferol 3-(6G-malonylneohesperidoside). Antioxidant testing with DPPH at concentrations of 10, 50, and 100 ppm revealed the Soxhlet extract had a stronger activity (IC_{50} = 14.328 ppm) compared to the macerated extract (IC_{50} = 32.092 ppm), with statistically significant differences ($p < 0.05$). Molecular docking demonstrated that Kaempferol 3-(6G-malonylneohesperidoside) exhibited the strongest binding affinity to NADPH oxidase (-10.1 kcal/mol), followed by other flavonoid derivatives. These findings underscore the antioxidant potential of M. oleifera, particularly from Soxhlet extraction, and suggest its promising application in pharmaceutical development as a natural antioxidant source.

Keywords: *Antioxidant activity, In silico studies, metabolomic profiling,*

Graphical Abstract



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DOI: https://doi.org/10.22437/chp.v9i1.36870
Received August 08th 2024; **Accepted** May 28th 2025; **Available online** June 01St 2025
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Introduction

The rich abundance and diversity of natural resources in Indonesia have resulted in a lack of understanding among the Indonesian population regarding the optimal utilization of these natural products, leading them to favor the consumption of instant foods instead [1]. Unhealthy lifestyles can cause the body to be continuously exposed to free radical compounds [2]. Changes in the human body are often exposed to dangerous substances that can cause disease and degenerative changes. Most diseases begin with excessive oxidation reactions in the human body [3]. Cancer, organ cell damage, cataracts, and degenerative diseases are some examples of cell damage caused by free radicals [4]. Other examples are pollution, alcohol, tobacco smoke, heavy metals, transition metals, industrial solvents, pesticides, certain drugs, and radiation [5].

Antioxidants are one way to prevent free radicals in the body. Natural ingredients in plant parts such as roots, stems, leaves, flowers, fruit, seeds, and pollen can contain antioxidants [6]. The moringa leaf has the potential to be an antioxidant. Moringa leaves contain more than forty types of natural antioxidants, making them a valuable source of natural antioxidant plants with secondary metabolite compounds that contain flavonoids, tannins, alkaloids, and saponins [7]. Similarly, reported the antioxidant activity of a 70% ethanol extract of Moringa leaves (*Moringa oleifera L.*) on DPPH; it shows that Moringa leaves contain flavonoid compounds that are useful as antioxidants, as proven by the results of the phytochemical screening carried out [8]. This was supported by results from another study, which showed that the ethanol extract of Moringa leaves has antioxidant activity with an IC.50 value of 18.15 μg/mL [9].

The antioxidant compounds in Moringa leaves can be extracted using several methods. Maceration and soxhletation extraction methods were chosen to extract Moringa leaves because they have many advantages over other extraction methods. The main advantage of the maceration extraction method is that the procedures and equipment used are simple; the maceration extraction method is not heated so that natural materials do not decompose. Cold extraction allows many compounds to be extracted, but some compounds have limited solubility in the extraction solvent at room temperature. Meanwhile, the hot extraction method (soxhletation) is the best method for obtaining extensive extract results. Also, it uses less solvent, the extraction process is fast, and it allows for complete sample extraction because the process is repeated. In addition, because the biological activity is not lost when heated, this technique can be used to search for drug-relevant compounds [10].

Herein, the effect of maceration and soxhletation extraction on the antioxidant content of *Moringa oleifera L.* leaves was analyzed using LC-MS with ethanol solvent. The antioxidant potential of the macerated and soxhleted *Moringa oleifera L.* leaf extracts was compared by DPPH test. The antioxidant potential of the main metabolites from the two extraction methods was evaluated in silico through molecular docking with NADPH Oxidase enzyme, using Apocynin A as a positive control. The investigation of the *Moringa oleifera* L species opens perspectives towards a better understanding of its biological efficiency and its interpretation based on the chemical composition of the studied secondary metabolites.

Materials and Methods

Materials

The study used samples of Moringa leaves from Pecuk Village, Pakel District, Tulungagung Regency, Indonesia. Chemicals like aquadest, Vitamin C (ascorbic acid), ethanol 96% (ABSOLUTE), Mayer's reagent, Dragendorff's reagent, Wagner's reagent, hydrochloric acid (HCl) (EMSURE®), Zn powder, ferric chloride ($FeCl_3$), chloroform, anhydrous acetic acid, sulphuric acid (H_2SO_4) and DPPH powder (1,1-diphenyl-2-picrylhydrazyl (HIMEDIA®), distilled water (ABSOLUTE).

Preparation and Extraction

The initial step involved selecting mature *Moringa oleifera* leaves, followed by wet sorting and air-drying under shade to prevent degradation of

active compounds. Extraction was performed using two methods: (1) **Maceration**—250 g of powdered simplicia was soaked in 1000 mL of 96% ethanol for 72 hours with periodic agitation to ensure diffusion equilibrium. (2) **Soxhletation**—50 g of powdered simplicia was extracted using a Soxhlet apparatus with 500 mL of 96% ethanol at 40–55 °C. Both extracts were concentrated using a water bath at the same temperature range until a thick extract was obtained

Phytochemical screening

Phytochemical screening of the *Moringa oleifera* L. ethanolic extract was conducted using standard qualitative methods. Phenols were detected by the addition of $FeCl_3$ solution, indicated by a green or blackish-blue color change. Flavonoids were identified by reacting the thick extract with magnesium powder and 2% HCl, resulting in an orange-red coloration if positive. For alkaloid detection, the extract was mixed with 2N HCl and distilled water, heated in a water bath, filtered, and reacted with Mayer's reagent; the formation of a brownish-yellow precipitate indicated a positive result [11]. Moreover, saponins were tested by adding the extract to cold and hot water, shaking for 10 seconds, and adding a drop of 2N HCl; the formation of stable foam persisting for 10 minutes indicated the presence of saponins. Tannins were confirmed by adding 1–2 drops of 1% $FeCl_3$ to the extract, with a green or blackish-blue color change suggesting a positive result [11]. Terpenoids were screened by mixing the extract with chloroform, acetic anhydride, and concentrated sulfuric acid; the appearance of a red or purple coloration and the formation of a brownish ring indicated terpenoid presence [12]-[13].

Metabolites Profilling by LC-MS

The solvent-free extract was dissolved using pro-analysis methanol to a concentration of 20 ppm with a sample ratio 1:5. Samples were taken at 1 μL and injected into the LC-MS instrument system. The liquid chromatography is coupled with a mass spectrometry of a quadrupole time-of-flight (QTOF) system with a positive ionization mode. The analysis conditions used are column Shimadzu Shim Pack FC-ODS (2mm x 150mm, 3μm); flow rate 0.5 mL/min; injection volume 1 μL; solvent ethanol 95%; column temperature 35 °C. After get all metabolites profle then screen antioxidant activity used DPPH method.

Antioxidant Activity by DPPH assay

A 1 mL aliquot of 100 ppm DPPH in absolute ethanol was mixed with *Moringa oleifera* ethanolic extract and diluted to 5 mL. Absorbance was measured between 510–520 nm, and 515 nm was selected as the optimum wavelength. For extract preparation, 2.5 g of thick *Moringa oleifera* extract was dissolved in 5 mL ethanol to obtain a 1000 ppm stock solution. Antioxidant activity, including vitamin C comparison, was evaluated using the DPPH method. A 3 mL sample was combined with 2 mL DPPH solution and 2 mL ethanol in a 10 mL volumetric flask, incubated in the dark at room temperature for 30 minutes, and absorbance was measured at 515 nm. The percentage of inhibition was calculated using Equation (1), and IC_{50} values were derived from the linear regression equation (Equation 2), indicating the concentration required to inhibit 50% of DPPH radicals [1].

$$\%\ \text{inhibition} = \frac{(\text{blank abs} - \text{sample abs})}{\text{blank abs}} \times 100\% \quad (1)$$

$$\text{IC}_{50} = \frac{(50 - \text{a})}{\text{b}} \quad (2)$$

Molecular Docking of Potential Compounds toward NADPH Oxidase

The preparation of ligand structures involved converting the two-dimensional (2D) molecular structures of five major compounds identified via LC-MS, drawn using ChemDraw Ultra 22.0, into three-dimensional (3D) models using Chem3D 22.0, saved in PDB format. Hydrogen atoms were added using Discovery Studio 2021, and the ligands were saved in PDF format. Subsequently, ligand optimization and torsion bond settings were performed with AutoDockTools, and the ligands were saved in pdbqt format.

The macromolecular structure of NADPH oxidase (PDB ID: 4Z3D) was retrieved from the Protein

Data Bank (https://www.rcsb.org). UCSF Chimera was used to remove solvents, native ligands, and nonstandard residues. After cleanup, the macromolecule was saved in PDB format. Optimization was performed using AutoDockTools by adding Kollman charges and hydrogen atoms, and the receptor was saved in pdbqt format. Molecular docking was carried out using PyRx software based on AutoDockTools. Optimized ligand and receptor files were placed in a single folder. A flexible docking protocol was employed with grid box parameters set according to validation results. Docking was performed using the AutoDock Wizard feature in PyRx, and results were presented as binding affinity values and interaction data. Final docking poses were saved in PDB format. Visualization and analysis of docking results were performed using Discovery Studio Visualizer 2021, allowing detailed observation of receptor-ligand interactions.

Result and Discussion

Phytochemical screening was conducted to identify the presence of secondary metabolites in *Moringa oleifera* L. leaf ethanolic extracts by observing specific colorimetric changes following the addition of chemical reagents [14]. The results, summarized in Table 1, indicate the successful identification of several bioactive compound classes.

Table 1. Moringa leaf extract phytochemical screening test results

Compound Classes	Reagents	Discolorations	Results	
			Maceration	Soxhletation
Phenols	$FeCl_3$	Green	+	+
Flavonoids	Mg + HCl 2%	Red	+	+
Alkaloids	Weagner	Chocolate	+	+
	Dagendroft	Orange	+	+
Tannins	$FeCl_3$ 1%	Green	+	+
Saponins	H_2O (hot) + HCl 2 N	Foam	-	+
Terpenoids	$C_4H_6O_3 + H_2SO_4$	Red ring	+	+

Notes : (+) = contains the test compound ; (-) = does'nt contain the test compound

Phenolic compounds were positively identified in both macerated and soxhletated extracts, as evidenced by a blackish-green color upon reagent addition. Flavonoids were also present in both extracts, indicated by a red color change. The addition of concentrated HCl facilitates hydrolysis of O-glycosylated flavonoids into their aglycone forms, while magnesium and acid reduce the compounds to produce red-colored complexes such as flavones, flavanones, and xanthones [15]. Alkaloid presence was confirmed using Wagner and Dragendorff reagents. Both tests yielded positive results for alkaloids in the extracts by showing precipitates [16]. Moreover, Tannin compounds were also detected in both extracts, signaled by a blackish-green coloration upon the addition of $FeCl_3$, indicating the formation of tannin–Fe^{3+} complexes [17].

Furthermore, saponins were detected only in the soxhletation extract, evidenced by stable foam formation, suggesting that heat-assisted extraction facilitates the release of these glycosidic compounds. Conversely, maceration failed to extract saponins, likely due to the milder, cold conditions. Both extraction methods yielded positive results for terpenoids, confirmed through the Liebermann–Burchard reaction, indicated by a red color and occasional brown ring formation [18,19].

Metabolites Profilling by LC-MS

Based on the results of LC-MS analysis, the maceration method obtained the separation results of 101 compounds (Figure 1) that have the potential as antioxidants and have been grouped according to the secondary metabolite group with a total percentage composition of flavonoids (70.9872%), phenols (8.09702%), alkaloids (3.35195%), tannins (2.0379%), and terpenoids (0.40774%).

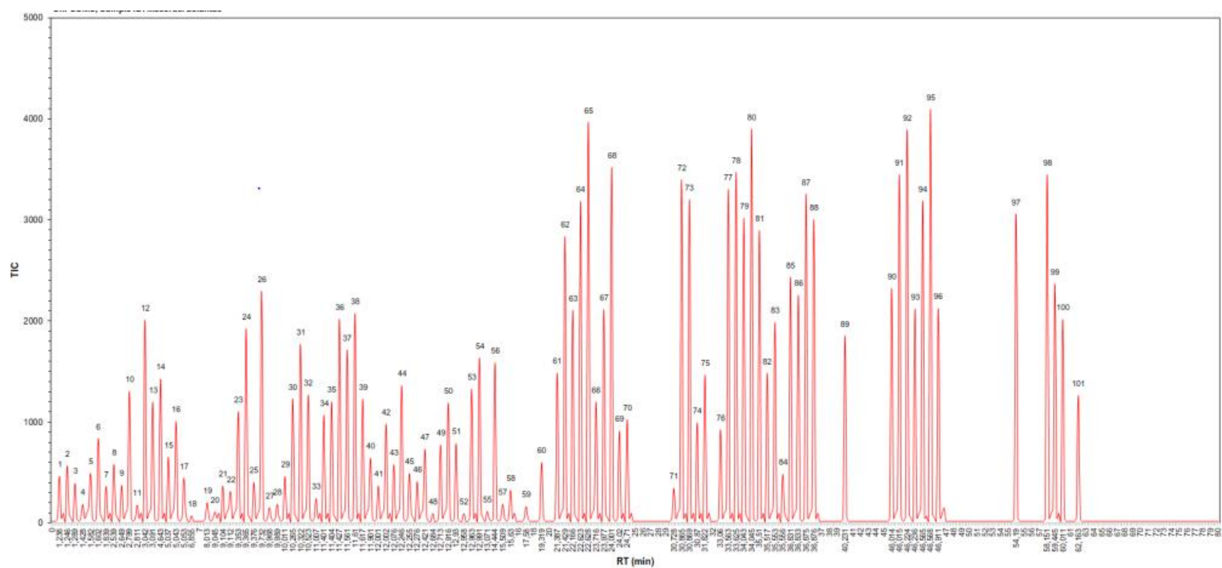

Figure 1. LCMS Chromatogram Results of the *Moringa oleifera* L. macerate Analysis conditions: column Shimadzu Shim Pack FC-ODS (2mm x 150mm, 3μm); flow rate 0.5 mL/min; injection volume 1 μL; solvent ethanol 95%; column temperature 35 ºC

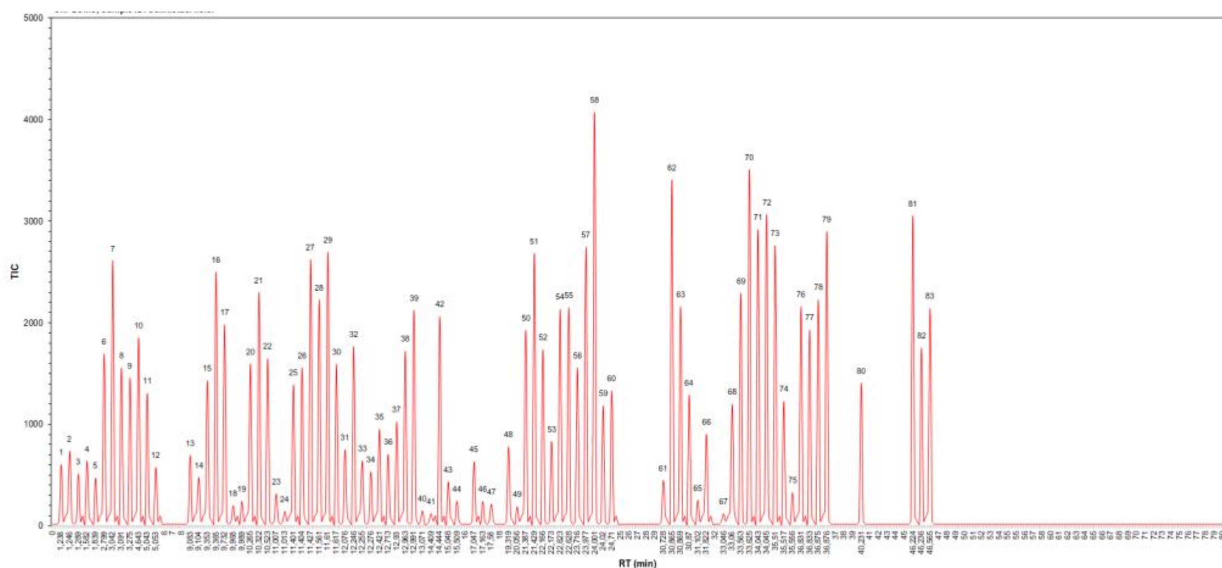

Figure 2. LCMS Chromatogram Results of the *Moringa oleifera L.* soxhlet extract. Analysis conditions: column Shimadzu Shim Pack FC-ODS (2mm x 150mm, 3μm); flow rate 0.5 mL/min; injection volume 1 μL; solvent ethanol 95%; column temperature 35 ºC.

Table 2 presents the LC-MS-based profiling of secondary metabolites in *Moringa oleifera* L. leaf extracts, including phenolics, flavonoids, alkaloids, tannins, terpenoids, and saponins. A total of eight major flavonoid derivatives were identified. Peaks 72, 80, 81, and 92 appeared consistently in both maceration and soxhletation extracts, with retention times ranging from 22.6 to 58.1 minutes, indicating their stability across extraction methods. Peaks unique to the soxhlet extract included quercetin-3-O-glucoside (m/z 463.37), kaempferol-3-O-(6"-malonylglucoside) (m/z 534.42), and kaempferol 3-neohesperidoside (m/z 594.52), each confirmed via characteristic fragmentation spectra. The maceration extract, on the other hand, revealed the presence of luteolin-7-glucoside (peak 65, m/z 448.10), a high molecular weight glycoside (peak 95, m/z 756.66), and ternatin C3 (peak 98, m/z 1021.86), a bioactive anthocyanin (Figure 2).

Peaks 72/80 and 81/92 were tentatively assigned as kaempferol 3-O-robinobioside and kaempferol 3-(6G-malonylneohesperidoside), respectively, supported by their identical m/z values (594.52 and 680.56) and shared fragmentation behavior. The detection of these compounds in both extraction techniques underscores their abundance and potential pharmacological relevance. Previous studies have also reported similar compounds in *M. oleifera*, demonstrating significant antioxidant, anti-inflammatory, and antidiabetic properties [12,15,20]. The findings confirm the utility of LC-MS in identifying bioactive flavonoid glycosides and support *M. oleifera* as a rich source of functional phytochemicals.

Antioxidant Activity

The optimum wavelength of DPPH was 515 nm (absorbance 0.635) and used to calculate % inhibition and IC_{50} via linear regression. Antioxidant activity of Moringa leaf extract and vitamin C was evaluated based on IC_{50}; lower IC_{50} indicates higher antioxidant potential in reducing DPPH radicals [20]. IC_{50} can be calculated by drawing a linear regression curve between % inhibition with the y-axis and the concentration series as the x-axis.

The linear regression equations obtained for *Moringa oleifera* leaf extracts from maceration and soxhletation methods were $y = 0.1916x + 43.851$ and $y = 0.1585x + 47.729$, respectively. For vitamin C, used as a positive control, the equation was $y = 10.788x - 3.1497$ (Figure 3). These equations were used to calculate the IC_{50} values, defined as the concentration required to inhibit 50% of DPPH radicals. The results demonstrated that both extraction methods yielded extracts with very strong antioxidant activity, comparable to that of vitamin C.

The IC_{50} values obtained were 32.092 ppm for the macerated extract, 14.328 ppm for the soxhlet extract, and 4.927 ppm for vitamin C. Based on the classification of antioxidant potency, where $IC_{50} < 50$ ppm indicates very strong activity, all three samples fall into this category. These findings suggest that Moringa oleifera leaf extracts, particularly those obtained via soxhletation, possess significant potential as natural antioxidants capable of mitigating oxidative stress caused by free radicals.

Statistical analysis using a paired t-test revealed a significant difference in antioxidant activity between the two extraction methods, with a p-value of 0.000 ($p < 0.05$), confirming that the soxhlet method yields a more potent antioxidant extract. This is further supported by LC-MS profiling, which showed higher flavonoid content—compounds known for their antioxidant properties—in the soxhlet extract (75.61%) compared to the maceration extract (70.99%). The high flavonoid content contributes to the superior antioxidant capacity of the soxhlet-derived extract, reinforcing its potential application in pharmaceutical or nutraceutical formulations.

Flavonoids are a prominent class of phytochemicals and represent the most abundant polyphenolic compounds found in fruits and vegetables. They possess strong antioxidant properties, which are expressed through multiple mechanisms, including scavenging reactive oxygen species (ROS) and free radicals, chelating transition metals, and inhibiting the oxidation of low-density lipoproteins (LDL). Flavonoids can donate hydrogen atoms to lipid radicals, forming more stable antioxidant derivatives less prone to further oxidation. Their antioxidant effects are mediated by direct ROS neutralization, metal ion chelation (as seen in quercetin's iron-binding properties), inhibition of ROS-generating enzymes, and upregulation of endogenous antioxidant enzymes. These pathways may act synergistically to enhance protective effects. The soxhletation extraction method, which utilizes continuous heating and solvent recycling, is particularly effective in isolating thermally stable flavonoids. It also facilitates the release of low molecular weight bioactives from complex polymer matrices, thereby optimizing the extraction of flavonoid compounds from plant materials like *Moringa oleifera* [24].

Table 2. Phenolic, flavonoids, alkaloids, tannins, terpenoids, and saponins derivatives identified in Moringa oleifera L. extract using LC-MS

Peak numbers	RT (min)	Composition (%)	Measured (*m/z*)	Formulas	MS/MS Fragments	Proposed Metabolites	Extracts
Phenolic compounds							
1	1.238	0.31609* 0.50197**	116.0760	$C_4H_4O_4$	117.0143, 116.0110	Fumaric acid	M, S
3	1.289	0.26828* 0.42605**	122.1230	$C_7H_6O_2$	123.0401, 122.0368	Benzoic acid	M, S
5 4	1.582	0.33379* 0.53008**	148.1610	$C_9H_8O_2$	149.0558, 148.0524	Cinnamic acid	M, S
7 5	1.839	0.24758* 0.39318**	164.1600	$C_9H_8O_3$	165.0507, 164.0473	p-coumaric acid	M, S
10 6	2.799	0.88305* 1.40236**	168.1480	$C_8H_8O_4$	169.0456, 168.0423	Vanillic acid	M, S
12 7	3.042	1.36068* 2.16088**	170.1200	$C_7H_8O_5$	172.0258, 171.0249, 170.0215	Gallic acid	M, S
13 8	3.091	0.81151* 1.42569**	174.1520	$C_7H_{10}O_5$	176.0571, 175.0562, 174.0528	Shikimic acid	M, S
14 10	4.643	0.96673* 1.53525**	180.1590	$C_9H_8O_4$	181.0456, 180.0423, 180.1590	Caffeic acid	M, S
41	14.409	0.10150	416.6900	$C_{28}H_{48}O_2$	418.3721, 418.3721, 417.3688, 416.3654	γ-tocopherol	S
16 11	5.043	0.68199* 1.08305**	194.1860	$C_{10}H_{10}O_4$	195.0613, 194.0579	Ferulic acid	M, S
45 33	12.255	0.33420* 0.53074**	336.2960	$C_{16}H_{16}O_8$	338.0912, 338.0888, 337.0879, 336.0845	5-O-caffeoylshikimic acid	M, S
46 34	12.276	0.27924* 0.44346**	338.1002	$C_{16}H_{18}O_8$	340.1069, 340.1044, 339. 1035, 338.1002	3-p-coumaroylquinic acid	M, S
47 35	12.421	0.49636* 0.78827**	354.0951	$C_{15}H_{18}O_8$	356.1018, 356.0993, 355.0984, 354.0951	Chlorogenic acid	M, S
49	20.056	0.15834	430.7170	$C_{29}H_{50}O_2$	432.3878, 432.3878, 431.3844, 430.3811	α-tocopherol	S

Peak numbers	RT (min)	Composition (%)	Measured (*m/z*)	Formulas	MS/MS Fragments	Proposed Metabolites	Extracts
59 47	17.58	0.11287* 0.17925**	425.4230	$C_{14}H_{18}NO_{19}S_2$	428.0442, 427.0517, 427.0493, 427.0408, 426.0484, 425.0450	4-hydroxybenzyl glucosinolate	M, S
61 50	21.387	1.00465* 1.59548**	432.3810	$C_{21}H_{20}O_{10}$	434.1099, 434.1124, 433.1090, 432.1056	Vitexin	M, S
Flavonoids derivatives							
9	3.275	1.20730	480.3780	$C_9H_6O_4$	179.0300, 178.0266, 178.1430	Escuetin	S
14	9.104	0.39765	268.2680	$C_{16}H_{12}O_4$	270.0803, 269.0769, 268.0736	3-hydoxy-4'-methoxyflavone	S
17	9.732	1.6399	279.1107	$C_{15}H_{12}NO_5$	274.0727, 274.0752, 273.0718, 272.0685	Naringenin	S
19	8.013	0.13799	254.2410	$C_{15}H_{10}O_4$	256.0646, 255.0613, 254.0579	Daidzein	M
21	9.104	0.25040	268.2680	$C_{16}H_{12}O_4$	270.0803, 269.0769, 268.0736	3-hydroxy-4'-methoxyflavone	M
21	10.322	1.90336	286.2390	$C_{15}H_{10}O_5$	288.0544, 288.0520, 287.0511, 286.0477	Kaemferol	S
22	9.112	0.21303	268.2680	$C_{16}H_{12}O_4$	270.0803, 269.0769, 268.0736	Formononetin	M
22	10.523	1.36443	298.2500	$C_{16}H_{10}O_6$	300.0520, 300.0544, 299.0511, 298.0477	2'.5-dihydroxy-6-7-methylenedioxyisoflavone	S
23 15	9.353	0.74657* 1.18561**	270.2810	$C_{13}H_{18}O_6$	272.1146, 271.1137, 270.1103	Benzyl-β-D-glucopyranoside	M, S
24	9.365	1.30251	270.2400	$C_{15}H_{10}O_5$	272.0571, 272.0595, 271.0562, 270.0528	Apigenin	M
25	9.376	0.27419	270.2400	$C_{15}H_{10}O_5$	272.0571, 272.0595, 271.0562, 270.0528	Genistein	M
26	0.732	1.55322	272.2560	$C_{15}H_{12}O_5$	274.0727, 274.0752, 273.0718, 272.0685	Narigenin	M

Peak numbers	RT (min)	Composition (%)	Measured (*m/z*)	Formulas	MS/MS Fragments	Proposed Metabolites	Extracts
26	11.404	1.29152	302.0790	$C_{16}H_{14}O_6$	304.0833, 304.0857, 303.0824, 302.0790	5,2',3'-trihydroxy-7-methoxyflavanone	S
27 18	9.968	0.10490* 0.16659**	279.2920	$C_{14}H_{17}NO_5$	281.1149, 281.1174, 280.1140, 279.1107	Niazirin	M, S
28	11.561	1.84376	312.2770	$C_{17}H_{12}O_8$	314.0676, 314.0701, 313.0667, 312.0634	2'-hydrox-5-methoxy-6,7-methylenedioxyisoflavone	S
29	10.011	0.31347	284.2670	$C_{16}H_{12}O_5$	286.0727, 286.0752, 285.0718, 284.0685	Biochanin A	M
29	11.61	2.23146	316.0583	$C_{16}H_{12}O_7$	318.0650, 318.0625, 317.0617, 316.0583	5,2'3'-trihyhydroxy-6,7-methylenedioxyisoflavone	S
30 20	10.265	0.83243* 1.32197**	286.2390	$C_{15}H_{10}O_6$	288.0544, 288.0520, 287.0511, 286.0477	Luteolin	M, S
31	10.322	1.19853	286.2390	$C_{15}H_{10}O_5$	288.0544, 288.0520, 287.0511, 286.0477	Kaempferol	M
31	12.076	0.62378	321.3290	$C_{16}H_{19}NO_5$	323.1255, 323.1279, 322.1246, 321.1212	Niazirinin	S
32	10.523	0.85917	298.2500	$C_{16}H_{10}O_6$	300.0520, 300.0544, 299.0511, 298.0477	2'-5-dihydroxy-6,7-methylenedioxyisoflavone	M
32	12.246	1.46440	624.5480	$C_{28}H_{32}O_{16}$	334.0938, 334.0963, 333.0930, 332.0896	3,5,3'-trihydroxy-7,2'-dimethoxyflavanone	S
34 25	11.401	0.72424* 1,15015**	302.1940	$C_{14}H_6O_8$	304.0130, 304.0105, 303.0096, 302.0063	Ellagic acid	M, S
35	11.404	0.81325	302.0790	$C_{16}H_{14}O_6$	304.0833, 304.0857, 303.0824, 302.0790	5,2',3'-trihydroxy-7-metoxyflavonone	M
36 27	11.427	1.36722* 2.17126**	302.2380	$C_{15}H_{10}O_7$	304.0494, 304.0469, 303.0460, 302.0427	Quercetin	M, S
37	11.561	1.16099	312.2770	$C_{17}H_{12}O_8$	314.0676, 314.0701, 313.0667, 312.0634	2'-hydroxy-5-methoxy-6,7-methylenedioxyisoflavone	M
38	11.61	1.40512	316.0583	$C_{16}H_{12}O_7$	318.0650, 318.0625, 317.0617, 316.0583	5,2',3'-trihidroxy-6-7-methylendioxyflavonone	M

Peak numbers	RT (min)	Composition (%)	Measured (*m/z*)	Formulas	MS/MS Fragments	Proposed Metabolites	Extracts
39 30	11.617	0.83070* 1.3922**	316.2650	$C_{15}H_{12}O_7$	318.0650, 318.0625, 317.0617, 316.0583	5,6,7,4'-tetrahydroxy-8-methoxyisoflavone	M, S
39	12.991	1.75841	386.3560	$C_{20}H_{18}O_8$	388.1044, 388.1069, 387.1035, 386.1002	5,3',4',5'-tetramethoxy-6-7-methylemdioxyisoflavone	S
40	11.901	0.43559	306.2700	$C_{15}H_{14}O_7$	308.0807, 308.0782, 307.0773, 306.0740	Leucocyanidin	M
41	12.001	0.24987	316.2650	$C_{16}H_{12}O_7$	318.0650, 318.0625, 317.0617, 316.0583	Rhamnetin	M
42	12.002	0.66523	316.2650	$C_{15}H_{12}O_7$	318.0650, 318.0625, 317.0617, 316.0583	Isorhamnetin	M
42	14.444	1.70645	402.3960	$C_{18}H_{26}O_{10}$	404.1593, 404.1568, 403.1560, 402.1526	benzyl-6-O-β-D-xylopyranosyl -β-D-glucopyranoside	S
44	12.246	0.92211	332.3080	$C_{17}H_{16}O_7$	334.0938, 334.0963, 333.0930, 332.0896	3,5,3'-trihydroxy-7,2'-dimethoxyflavonone	M
48	12.684	0.06654	361.3800	$C_{10}H_{19}NO_9S_2$	362.0495, 363.0544, 363.0459, 362.0535, 361.0501	Glucoputranjivin	M
49 36	12.713	0.52454* 0.58500**	366.2960	$C_{15}H_{10}O_9$	368.0113, 368.0088, 368.0003, 367.0079, 366.0046	Kaempferol-3-O-sulfate	M, S
51	12.93	0.53432	376.3170	$C_{18}H_{16}O_9$	378.0861, 378.0837, 377.0828, 376.0794	(6R,6aS,12aR)-6,9,11,11a-tetrahydroxy-2,3-dimethoxyrotenone	M
51	21.429	2.22057	432.3810	$C_{21}H_{20}O_{10}$	434.1099, 434.1124, 433.1090, 432.1056	Kaempferol-3-rhamnoside	S
52	12.985	0.06521	376.3690	$C_{17}H_{20}N_4O_6$	378.1425, 377.1353, 378.1450, 377.1416, 376.1383	Riboflavin	M
53 38	12.963	0.89774* 1.42569**	377.3930	$C_{19}H_{23}NO_7$	379.1517, 379.1542, 378.1508, 377.1475	Pyrrolemarumine 4'-O-α-L-rhamnopyranoside	M, S

Peak numbers	RT (min)	Composition (%)	Measured (*m/z*)	Formulas	MS/MS Fragments	Proposed Metabolites	Extracts
53	22.173	0.69167	448.1006	$C_{21}H_{20}O_{11}$	450.1048, 450.1073, 449.1039, 448.1006	Astragalin	S
54	12.991	1.10725	386.3560	$C_{20}H_{18}O_8$	388.1044, 388.1069, 387.1035, 386.1002	5,3',4',5'-tetramethoxy-6,7-methylemedioxyisoflavone	M
54	22.623	1.76712	448.3800	$C_{21}H_{20}O_{11}$	450.1048, 450.1073, 449.1039, 448.1006	Kaempferol-3-O-glucoside	S
56	14.444	1.07453	402.3960	$C_{18}H_{26}O_{10}$	404.1593, 404.1568, 403.1560, 402.1526	Benzyl-6-O-b-D-xylopyranosyl-b-D-glucopyranoside	M
61	30.728	0.37250	530.4391	$C_{25}H_{22}O_{13}^{2-}$	266.0557, 266.0569, 265.5552, 265.0536	biochanin A-7-O-β-D glucoside-6"-O-malonate	S
62	21.429	1.91885	432.3810	$C_{21}H_{20}O_{10}$	434.1099, 434.1124, 433.1090, 432.1056	Kaemferol-3-rhamnoside	M
62	30.865	2.82097	534.4260	$C_{24}H_{22}O_{14}$	536.1052, 536.1077, 535.1043, 534.1010	Kaempferol-3-O-(6"-malonylglucoside)	S
63 52	22.166	1.42594* 1.43779**	466.4080	$C_{22}H_{22}O_{10}$	448.1255, 448.1280, 447.1247, 446.1213	Calycosin-7-O-β-D-glucoside	M, S
63	30,869	1.78668	534.4260	$C_{24}H_{22}O_{14}$	536.1052, 536.1077, 535.1043, 534.1010	luteolin-7-O-(6''-malonylglucoside)	S
64	22.623	2.15389	448.3800	$C_{21}H_{20}O_{11}$	450.1048, 450.1073, 449.1039, 448.1006	kaemferol-3-O-glucoside	M
65 55	22.628	2.68230* 1.77956**	448.1006	$C_{21}H_{20}O_{11}$	450.1048, 450.1073, 449.1039, 448.1006	Luteolin-7-glucoside	M, S
67 57	23.977	1.43035* 2.27152**	462.4070	$C_{22}H_{22}O_{11}$	464.1205, 464.1229, 463.1196, 462.1162	Hirsutrin	M, S
67	33.046	0.10184	550.8860	$C_{46}H_{56}O_2$	570.4347, 569.4314, 568.4280	Lutein	S
68 58	24.001	2.38175* 3.36906**	463.3715	$C_{21}H_{19}O_{12}$	465.0949, 465.0924, 464.0916, 463.0882	Quercetin-3-O-glucoside	M, S

Peak numbers	RT (min)	Composition (%)	Measured (*m/z*)	Formulas	MS/MS Fragments	Proposed Metabolites	Extracts
69 59	24.02	0.61754* 0.98071**	464.3790	$C_{21}H_{20}O_{12}$	466.1022, 466.0997, 465.0988, 464.0955	Hyperoside	M, S
70 78	33.625	2.90214* 2.34802**	594.5220	$C_{27}H_{30}O_{15}$	596.1652, 596.1652, 596.1627, 595.1618, 594.1585	Kaempferol 3-neohesperidoside	M, S
71	20.728	0.23456	530.4391	$C_{25}H_{22}O_{13}^{2-}$	266.0557, 266.0569, 265.5552, 265.0536	Biochanin A-7-O-β-D-glucoside-6"-O-malonate	M
72	30.865	0.23456	530.4391	$C_{24}H_{22}O_{14}$	536.1052, 536.1077, 535.1043, 534.1010	Kaempferol-3-O-(6"-malonylglucoside)	M
73	30.869	2.16621	534.4260	$C_{24}H_{22}O_{14}$	536.1052, 536.1077, 535.1043, 534.1010	Luteolin-7-O-(6”-malonylglucoside)	M
74 64	30.87	0.67304* 1.06884**	534.4700	$C_{25}H_{26}O_{13}$	536.1416, 536.1441, 535.1407, 534.1373	6,9-dihydroxy-2,310-trimethoxy-6a,12a-didehydrorotenone 9-O-glucoside	M, S
75 66	31.822	0.99273* 0.74982**	550.0959	$C_{24}H_{22}O_{15}$	552.1001, 552.1026, 551.0992, 550.0959	Quercetin-3-O-(6"-malonylglucoside)	M, S
76	36.831	1.78863	624.1690	$C_{28}H_{32}O_{16}$	626.1733, 626.1757, 625.1724, 624.1690	5,7,4'-trihydroxy-6-methoxysoflavone 4'-O(6"-glucosylglucoside)	S
77 69	33.563	2.23489* 1.89575**	580.5390	$C_{27}H_{32}O_{14}$	582.1859, 582.1835, 581.1826, 580.1792	Naringin	M, S
77	36.833	1.59821	624.5480	$C_{28}H_{32}O_{16}$	626.1733, 626.1757, 625.1724, 624.1690	5,7,4'-trihydroxy-6-methoxysoflavone 7-O-glucoside-4'-O-glucoside	S
79 71	33.043	2,04108* 2.41469**	594.5220	$C_{27}H_{30}O_{15}$	596.1652, 596.1627, 595.1618, 594.1585	kaempferol 3-O-rutinoside	M, S
79	36.876	2.39915	626.5200	$C_{27}H_{30}O_{17}$	629.1559, 628.1550, 628.1550, 628.1525	Myricetin-3-O-rutinoside	S

Peak numbers	RT (min)	Composition (%)	Measured (*m/z*)	Formulas	MS/MS Fragments	Proposed Metabolites	Extracts
80 72	34.045	2.63787* 2.53572**	594.5220	$C_{27}H_{30}O_{15}$	596.1652, 596.1652, 596.1627, 595.1618, 594.1585	Kaempferol 3-O-robinobioside	M, S
80	40.231	1.16501	624.5480	$C_{28}H_{32}O_{16}$	657.1872, 656.1863, 656.1863, 656.1838	5,7,4'-trihydroxy-6,3'-dimethoxhisoflavone 7-O-(6''-glucosylglucoside)	S
81 73	35.51	1.95911* 2.28451**	610.5210	$C_{27}H_{30}O_{16}$	612.1601, 612.1576, 611.1567, 610.1534	Quercetin 3-O-neohesperidoside	M, S
81	46.224	2.52670	680.5680	$C_{30}H_{32}O_{18}$	683.1665, 682.1656, 682.1656, 682.1631, 681.1622, 680.1589	Kaempferol 3-(6G-malonyineohesperidoside)	S
82 74	35.517	1.00453* 1.01657**	610.5210	$C_{27}H_{30}O_{16}$	612.1601, 612.1601, 612.1576, 611.1567, 610.1534	Rutin	M, S
82	46.236	1.45227	696.5670	$C_{30}H_{32}O_{19}$	699.1614, 698.1605, 698.1605, 698.1580, 697.1571, 696.1538	Quercetin 3-(6''-malonylneohesperidoside)	S
83	46.565	1.77221	756.6630	$C_{33}H_{40}O_{20}$	759.2189, 758.2155, 758.2180, 757.2146, 756.2113	kaempferol 3-gucosyl-(1→2)-[glucosyl-(1→3)-rhamnoside]	S
85	36.831	1.64686	624.5480	$C_{28}H_{32}O_{16}$	626.1733, 626.1757, 625.1724, 624.1690	5,7,4'-trihydroxy-6-methoxyisoflavone 4'-O-(6''-glucosy;glucoside)	M
86	36.833	1.52695	624.1690	$C_{28}H_{32}O_{16}$	626.1733, 626.1757, 625.1724, 624.1690	5,7,4'-trihydroxy-6-methoxyisoflavone 7-O-4'-O-glucoside	M
87 78	36.375	2.20249* 1.84431**	626.5200	$C_{27}H_{30}O_{17}$	629.1559, 628.1550, 628.1525, 627.1517, 626.1483	Myricetin 3-neohesperidoside/ Myricetin 3-O-rutinoside	M, S

Peak numbers	RT (min)	Composition (%)	Measured (m/z)	Formulas	MS/MS Fragments	Proposed Metabolites	Extracts
89	40.231	1.25417	654.5740	$C_{29}H_{34}O_{17}$	657.1872, 656.1863, 656.1838, 655.1830, 654.1796	5,7,4'-trihydroxy-6,3'-dimethoxyisoflavone 7-O-(6''-glucosylglucoside)	M
90	46.014	1.57274	772.6620	$C_{33}H_{40}O_{21}$	775.2138, 774.2105, 774.2129, 773.2096, 772.2062	Quercetin 3-glucosyl-(1→2)-rhamnoside-7-glucoside	M
91	46.015	2.33244	772.6620	$C_{33}H_{40}O_{21}$	775.2138, 774.2105, 774.2129, 773.2096, 772.2062	Myricetin 3-(2G-rhamnosylrutinoside)	M
92 81	46.224	2.63219* 2.52670**	680.5680	$C_{30}H_{32}O_{18}$	683.1665, 682.1656, 682.1656, 682.1631, 681.1622	Kaempferol 3-(6G-malonylneohesperidoside)	M, S
93	46.236	1.43505	696.5670	$C_{30}H_{32}O_{19}$	699.1614, 698.1605, 698.1580, 697.1571, 696.1538	Quercetiin 3-(6''-malonylneoheseridoside)	M
94	46.565	2.15710	756.6630	$C_{33}H_{40}O_{20}$	759.2189, 758.2155, 758.2180, 757.2146, 756.2113	Kaempferol 3-glucosyl-(1→2)-[glucosyl-(1→3)-rhamnoside]	M
95	46.568	2.76758	756.6630	$C_{33}H_{40}O_{20}$	759.2189, 758.218, 758.213, 757.2146, 756.2113.	3-(((2S,3S,4R,5S,6R)-4,5-dihydroxy-3-(((2S,3S,4R,5R,6R)-3,4,5-trihydroxy-6-methyltetrahydro-2H-pyran-2- yl)oxy)-6-((((2R,3R,4S,5R,6R)-3,4,5-trihydroxy-6-methyltetrahydro-2H-pyran-2- yl)oxy)methyl)tetrahydro-2H- pyran-2-yl)oxy)-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4H-chromen-4-one	M

Peak numbers	RT (min)	Composition (%)	Measured (*m/z*)	Formulas	MS/MS Fragments	Proposed Metabolites	Extracts
98	58.151	2.33205	1021.8595	$C_{45}H_{49}O_{27}$	1022.2498, 1024.2532, 1023.2498, 1023.2523, 1022.2489, 1021.2456	Ternatin C3	M
Alkaloids derivatives							
4	1.428	0.12657	123.1110	$C_6H_5N0_2$	124.0354, 123.0320	Niacin	M
9	2.649	0.25482	164,1570	$C_5H_{12}O_5$	166.0727, 165.0718, 164.0685	Rhamnose	M
11	2.811	0.12266	169.1800	$C_8H_{11}NO_3$	170.0772, 169.0739	Pyrodixine	M
13	9.083	0.57727	268.0372	$C_{15}H_8O_5$	270.0414, 270.0439, 269.0405, 268.0372	Coumestrol	S
17 12	5.053	0.30224* 0.47998**	194.1860	$C_{10}H_{10}O_4$	195.061, 194.058	4-hydroxymellein	M, S
23	11.007	0.26358	297.3070	$C_{14}H_{19}NO_6$	299.1279, 299.1255, 298.1246, 297.1212	2-(4-(((2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyltetrahydro-2H-pyran-2yl)oxy)phenyl)acetimedic acid	S
24	11.013	0.12197	298.2500	$C_{16}H_{10}O_6$	300.0520, 300.0544, 299.0511, 298.0477	8-methoxycoumestrol	S
28 19	9.989	0.12753* 0.20253**	282.2510	$C_{16}H_{10}O_5$	284.0571, 284.0595, 283.0562, 282.0528	9-O-methylcoumestrol	M, S
33	11.007	0.16597	297.3070	$C_{14}H_{19}NO_6$	299.1279, 299.1255, 298.1246, 297.1212	2-(4-(((2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)phenyl)acetimidic acid	M

Peak numbers	RT (min)	Composition (%)	Measured (*m/z*)	Formulas	MS/MS Fragments	Proposed Metabolites	Extracts
37	12.93	0.84855	376.3170	$C_{18}H_{23}O_9$	378.0861, 378.0837, 377.0828, 376.0794	(6R,2aS,12aR)-6,9,11,11a-tetrahydroxy-2-3-dimethoxyrotenone	S
43	12.076	0.39278	321.3290	$C_{16}H_{19}NO_6$	323.1255, 323.1279, 322.1246, 321.1212	Niazinirin	M
50	12.916	0.80533	375.4070	$C_{11}H_{21}NO_9S_2$	378.0649, 376.0652, 377.0700, 377.0616	Glucocochlearin	M
55 40	13.071	0.07882* 0.12517**	391.4060	$C_{11}H_{21}NO_{10}S_2$	394.0598, 392.0601, 393.0649, 393.0565, 392.0640, 391.0607	Glucoconringiin	M, S
57 44	15.509	0.12791* 0.20314**	408.4165	$C_{14}H_{18}NO_9S_2^-$	410.0496, 411.0420, 409.0422, 410.0471, 410.0386, 409.0462, 408.0428	Benzyl glucosinolate	M, S
58	15.63	0.22133	411.4690	$C_{19}H_{25}NO_2S$	413.1394, 413.1419, 413.1310, 412.1385, 411.1352	Niaziminin B	M
75	35.556	0.27314	613.6020	$C_{22}H_{31}NO_{15}S_2$	614.1129, 616.1127, 615.1202, 615.1178, 615.1093, 614.1169, 613.1135	4-(4'-O-acetyl-α-L-rhamnopyranosyloxyl) benzylglukosinolate	S
76 68	33.06	0.62599* 0.99413**	571.5650	$C_{20}H_{29}NO_{14}S_2$	572.1023, 574.1021, 573.1097, 573.1072, 573.0987, 572.1063, 571.1029	4-(α-L-rhamnopyranosyloxy)-benzylglucosinolate	M, S
Tannis derivatives							
70 60	24.710	0.69410* 1.10229**	480.3780	$C_{21}H_{20}O_{13}$	482.0971, 482.0946, 481.0937, 480.0904	Isomyricitrin	M, S
83	35.553	1.34380	610.5240	$C_{30}H_{26}O_{14}$	612.1390, 612.1390, 612.1365, 611.1356, 610.1323	Prodelphinidin B1	M

Peak numbers	RT (min)	Composition (%)	Measured (*m/z*)	Formulas	MS/MS Fragments	Proposed Metabolites	Extracts
Terpenoids derivatives							
45	17.047	0.52445	426.6850	$C_{29}H_{46}O_2$	428.3565, 428.3565, 427.3531, 426.3498	Stigmast-4-en-3,6-dione	S
60 48	19.319	0.40774* 0.64753**	426.3862	$C_{30}H_{50}O$	428.3929, 428.3929, 427.3895, 426.3862	β-amyrin	M, S
65	31.102	0.21192	536.8880	$C_{40}H_{56}$	538.4449, 537.4416, 536.4382	β-carotene	S
Saponins derivatives							
43	15.048	0.36183	400.6910	$C_{28}H_{48}O$	402.3772, 402.3772, 401.3739, 400.3705	Campesterol	S
46	17.163	0.20177	480.3780	$C_{21}H_{20}O_{13}$	416.3929, 416.3929, 415.3895, 414.3862	β-sitosterol	S

Note: RT, retention time; M, maceration; S, soxhletation ; *, maceration; **, soxhletation

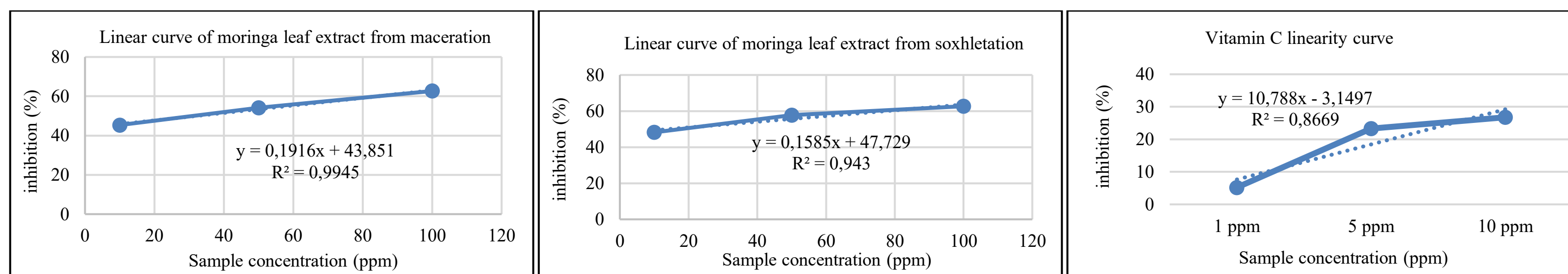

Figure 3. The curve of the relationship between active ingredient concentration and % inhibition. (A) Antioxidant activity of *Moringa oleifera* L. Macerate. (B) The antioxidant activity of the extract obtained from the soxhletation of Moringa oleifera L was examined. (C) antioxidant activity of vitamin C

The results of this study are in accordance with several other studies which states that the soxhlet method is slightly better than maceration. Including research to evaluate the effect of maceration and soxhletation extraction techniques on the antioxidant activity and total phenolic content of Bouea macrophylla Griff Plant [25]. Compare the maceration and soxhletation extraction methods on the antioxidant activity and total phenolic content of jackfruit leaves [26]. Determine the effect of maceration and soxhletation extraction methods on the levels of flavonoids in the leaves of the anting-anting [27].

Evaluation of Molecular Docking

Molecular docking was performed on five compounds of maceration method and five compounds of soxhlet method identified using LC-MS to identify potential binding modes of the compounds that could justify their inhibitory activity. Table 3 and Table 4 shows the binding affinity of the control ligand, the detected bioactive compound against NADPH Oxidase enzyme. The control docking procedure was performed using co-crystallized control ligands to validate the docking parameters. In this study, the complex with more negative values (i.e., stronger binding affinity) was considered as the best docked complex. The re-docked NADPH Oxidase was found to bind to 2CDU in a manner identical to its crystallographic configuration. The mean square deviation (RMSD) value of the re-docked NADPH Oxidase was found to be 0 Å, indicating that the selected docking parameters were able to reproduce the crystallized conformation. The docking parameters were considered acceptable if the RMSD value of the re-docked ligand, with respect to the crystallized one, was less than 1.9 Å [28]. Control docking showed that the control ligand exhibited a binding affinity of -9.8 kcal.mol to the enzyme. The superimposed 3D docking visualization (Figure 4 and Figure 5) depicts the predicted binding sites of the five identified compounds and the control ligand in the enzymatic protein. According to the figure, all the compounds are predicted to bind to the A domain of the enzyme, where the catalytic site is located. The same site is also occupied by the control ligand indicating that a similar inhibition mechanism might be followed by the identified compounds. Compound 1 is 3-(((2S,3S,4R,5S,6R)-4,5-dihydroxy-3-(((2S,3S,4R,5R,6R)-3,4,5-trihydroxy-6- methyltetrahydro-2H-pyran-2- yl)oxy)-6-((((2R,3R,4S,5R,6R)-3,4,5-trihydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)methyl)tetrahydro-2H- pyran-2-yl)oxy)-2-(3,4- dihydroxyphenyl)-5,7- dihydroxy-4H-chromen-4-one, compound 2 is luteolin-7-glucoside, compound 3 is Kaempferol 3-O-robinobioside, compound 4 is Kaempferol 3-(6G-malonylneohesperidoside), and compound 5, namely ternatin C3.

Table 3. Binding affinity of NADPH Oxidase enzyme (2CDU) with the control ligand of the identified active compound from the maceration method.

Compounds	Binding Affinity, kcal/mol
Control Ligand	-9.8
1	-9.0
2	-8.8
3	-9.6
4	-10.1
5	-6.9

Table 4. Binding affinity of NADPH Oxidase enzyme (2CDU) with the control ligand of the identified active compound from the soxhlet method.

Compounds	Binding Affinity, kcal/mol
Control Ligand	-9.8
1	-8.8
2	-9.6
3	-9.2
4	-9.6
5	-10.1

Table 5 shows the binding interactions of compounds 1, 2, 3, 4 and 5. Along with H-bonding interactions, other interactions like pi-sigma, pi-alkyl, pi-donor, t-shaped pi-pi and stracked amide-pi are also involved in the docked complexes. In the ligand complex against NADPH oxidase, the control ligand showed interactions involving hydrogen bonds with residues ASP179, SER157, GLY244, TYR188, GLY156, ILE160, and TYR159, as well as charge–charge and pi

interactions with LYS187, ILE243, and VAL214. Compound 1 interacted through hydrogen bonds with CSX42, ILE160, SER328, SER326, and TYR188, followed by pi-donor and pi–pi T-shaped interactions. Compound 2 formed strong hydrogen bonds with ASP179, GLY158, and TYR188, and showed pi-donor, pi–pi T-shaped, pi-alkyl, and amide–pi stacked interactions with residues PRO298 and ILE243. Compound 3 forms hydrogen bonds with GLY161, ILE160, CSX42, TYR188, PRO298, and CYS242, and interacts through pi-alkyl and pi-sigma bonds with TYR159, ALA300, and LEU299. Compound 4 forms hydrogen bonds with LYS213, ILE243, TYR159, CYS242, TYR188, SER326, GLY158, and GLY244, and pi-alkyl and pi-sigma interactions with ILE297 and PRO298. Compound 5 forms a number of hydrogen bonds with ALA300, CSX42, ASP282, LYS187, ARG246, and THR118, as well as pi-donor, pi-cation, and amide-pi stacked interactions with TYR188, ILE243, and LYS213, which overall indicate a strong binding affinity to the active site of NADPH oxidase.

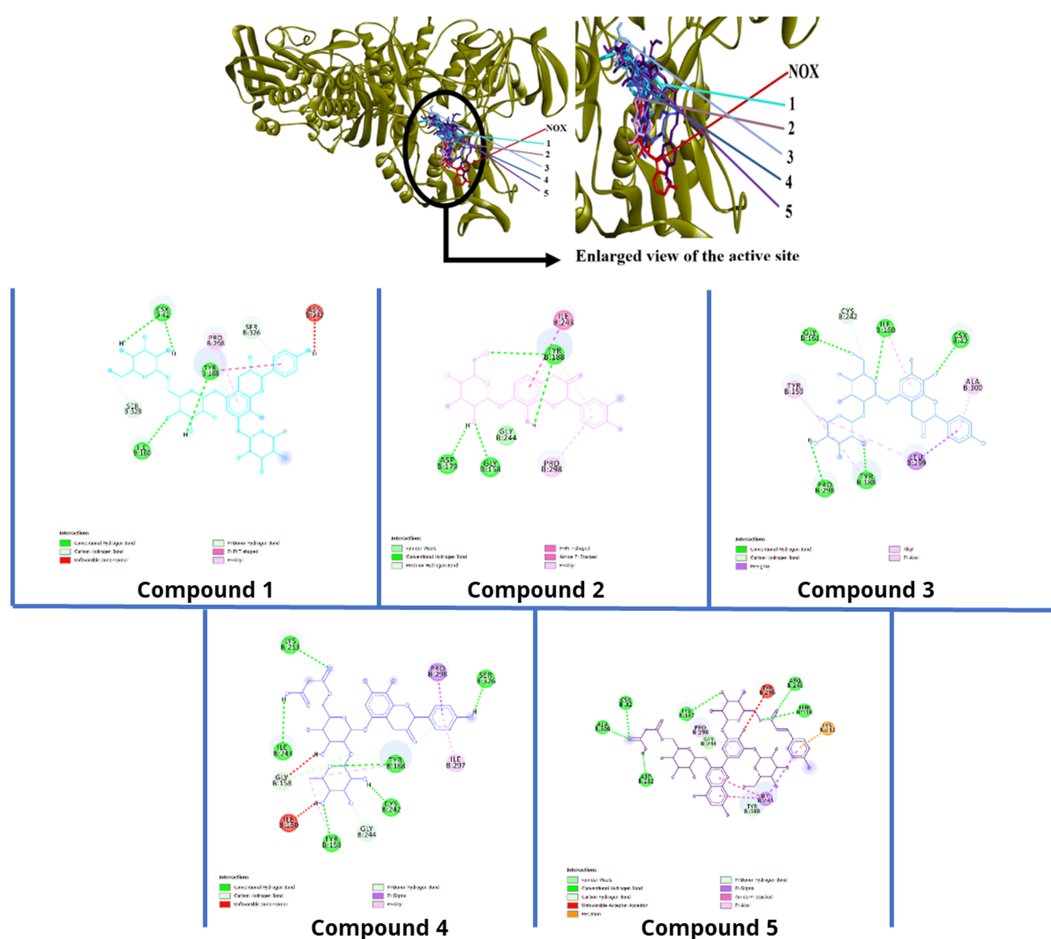

Figure 4. (A) Superimposed 3D diagram of control ligand, the docked compounds. (B) 2D binding interactions of the docked compounds from the maceration method with 2CDU.

Table 5. Data for the molecular docking of compounds 1, 2, 3, 4 and 5 from maceration method in 2CDU.

Compound Structure	Interacting Amino Acid Residues	Bonding Types	Bonding Distance (Å)
NADPH Oksidase **Ligand Control**	LYS213	Pi-Alkyl	4.15; 4.76
	ASP179	Hydrogen Bond	3.04; 3.20 3.24; 3.29

Compound Structure	Interacting Amino Acid Residues	Bonding Types	Bonding Distance (Å)
	SER157	Hydrogen Bond	3.23
	GLY244	Hydrogen Bond	3.42
	TYR188	Hydrogen Bond	2.55
	LYS187	Charge-Charge	5.47
	GLY156	Hydrogen Bond	3.65
	ILE160	Hydrogen Bond	3.38
	TYR159	Hydrogen Bond	3.06
	ILE243	Pi-Sigma Pi-Aklyl	3.39 4.97
	VAL214	Pi-Alkyl Hydrogen Bond Hydrogen Bond	5.22 2.72 3.13
3-(((2S,3S,4R,5S,6R)-4,5-dihydroxy-3- (((2S,3S,4R,5R,6R)-3,4,5-trihydroxy-6- methyltetrahydro-2H-pyran-2- yl)oxy)-6-((((2R,3R,4S,5R,6R)-3,4,5-trihydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)methyl)tetrahydro-2H- pyran-2-yl)oxy)-2-(3,4- dihydroxyphenyl)-5,7- dihydroxy-4H-chromen-4-one **Compound 1**	CSX42	Hydrogen Bond Hydrogen Bond	2.20 2.28
	ILE160	Hydrogen Bond	3.02
	TYR188	Hydrogen Bond Pi-Donor Pi-Pi T-shaped Pi-Pi T-shaped	2.49 3.27 4.65 5.64
	SER328	Hydrogen Bond	3.77
	SER326	Hydrogen Bond	3.19
	PRO298	Pi-Alkyl	4.52
luteolin-7-glucoside **Compound 2**	ASP179	Hydrogen Bond	2.47
	GLY158	Hydrogen Bond	2.83
	TYR188	Hydrogen Bond Hydrogen Bond Pi-Donor Hydrogen Bond Pi-Donor Hydrogen Bond Pi-Pi T-shaped	2.56 2.86 3.50 4.03 5.14 5.14 5.14
	PRO298	Pi-Alkyl	4.76
	ILE243	Amide Pi-Stacked	5.38
Kaempferol 3-O-robinobioside **Compound 3**	GLY161	Hydrogen Bond	3.07
	ILE160	Hydrogen Bond Pi-Alkyl	3.31 5.34
	CSX42	Hydrogen Bond	2.88
	TYR188	Hydrogen Bond Hydrogen Bond Pi-Alkyl	3.05 3.21 4.23
	PRO298	Hydrogen Bond	2.67
	TYR159	Pi-Alkyl	4.19
	CYS242	Hydrogen Bond	2.92
	ALA300	Pi-Alkyl	5.33
	LEU299	Pi-Sigma	3.31
Kaempferol 3-(6G-malonylneohesperidoside)	LYS213	Hydrogen Bond	3.07
	ILE243	Hydrogen Bond	2.62

Compound Structure	Interacting Amino Acid Residues	Bonding Types	Bonding Distance (Å)
Compound 4	TYR159	Hydrogen Bond Pi-Alkyl	2.80 4.80
	CYS242	Hydrogen Bond	2.16
	TYR188	Hydrogen Bond Pi-Donor Pi-Alkyl	3.10 3.83 4.37
	SER326	Hydrogen Bond	2.21
	GLY158	Hydrogen Bond	3.51 3.56
	GLY244	Hydrogen Bond	2.73
	ILE297	Pi-Alkyl	5.28
	PRO298	Pi-Sigma	3.95
Ternatin C3 **Compound 5**	ALA300	Hydrogen Bond	3.02
	CSX42	Hydrogen Bond	3.09
	ASP282	Hydrogen Bond	2.37
	LYS187	Hydrogen Bond	3.10
	ARG246	Hydrogen Bond	2.76
	THR118	Hydrogen Bond	3.37
	PRO298	Pi-Alkyl	4.84
	TYR188	Pi-Donor Hydrogen Bond Hydrogen Bond	2.89 4.07 4.07
	ILE243	Pi-Sigma Amide-Pi Stracked Amide-Pi Stracked	3.72 3.60 4.07 4.07 4.07
	LYS213	Pi-Cation Pi-Donor Pi-Alkyl	4.18 4.18 5.10

Table 6 shows the binding interactions of compounds 1, 2, 3, 4 and 5. Along with H-bonding interactions, other interactions like pi-sigma, pi-alkyl, pi-donor, t-shaped pi-pi and stracked amide-pi are also involved in the docked complexes. Compounds 1 to 5 showed strong interactions with the enzyme target through various bond types, such as hydrogen bonds, Pi-Pi, Pi-Alkyl, Pi-Sigma, and Amide-Pi Stacked. The most frequently interacting amino acid residues included TYR188, CSX42, TYR159, ILE160, and PRO298. In general, the hydrogen bond type was dominant, with the bond distance ranging from 1.98 to 5.95 Å. Compounds 1 and 4 formed many hydrogen and Pi-Pi bonds, while compounds 3 and 5 showed additional Pi-Donor and Amide-Pi Stacked interactions that strengthened the affinity to the target. These results indicated that all compounds had strong interaction potential through the contribution of various active residues and diverse bond types.

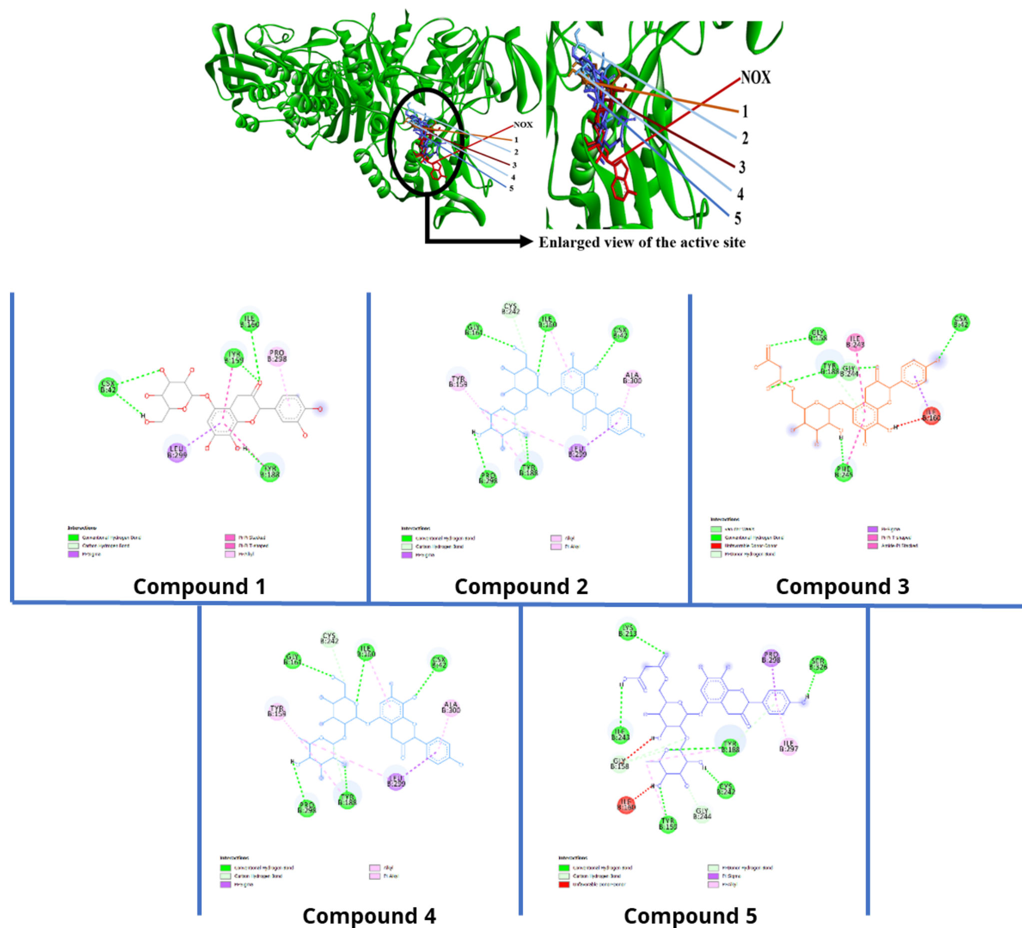

Figure 5. (A) Superimposed 3D diagram of control ligand, the docked compounds. (B) 2D binding interactions of the docked compounds from the soxhlet method with 2CDU.

Table 6. Data for the molecular docking of compounds 1, 2, 3, 4 and 5 from soxhlet method in 2CDU.

Compound Structure	Interacting Amino Acid Residues	Bonding Type	Bonding Distance (Å)
quercetin-3-O-glucoside **Compound 1**	CSX42	Hydrogen Bond	2.34
		Hydrogen Bond	2.90
		Hydrogen Bond	3.31
	TYR159	Hydrogen Bond	3.17
		Pi-Pi Stacked	4.22
	ILE160	Hydrogen Bond	3.36
	TYR188	Hydrogen Bond	2.15
		Pi-Pi T-shaped	5.62
	LEU299	Pi-Sigma	3.61
	PRO298	Pi-Alkyl	5.23
kaempferol 3-neohesperidoside **Compound 2**	GLY161	Hydrogen Bond	3.07
	ILE160	Hydrogen Bond	3.31
		Pi-Alkyl	5.34
	CSX42	Hydrogen Bond	2.88
	TYR188	Hydrogen Bond	3.05
		Hydrogen Bond	3.21
		Pi-Alkyl	4.23
	PRO298	Hydrogen Bond	2.67

Compound Structure	Interacting Amino Acid Residues	Bonding Type	Bonding Distance (Å)
	TYR159	Pi-Alkyl	4.19
	CYS242	Hydrogen Bond	2.92
	ALA300	Pi-Alkyl	5.33
	LEU299	Pi-Sigma	3.31
kaempferol-3-O-(6"-malonylglucoside) **Compound 3**	GLY158	Hydrogen Bond	2.98
	TYR188	Hydrogen Bond Hydrogen Bond Pi-Donor Hydrogen Bond	3.07 3.32 4.02
	CSX42	Hydrogen Bond	3.13
	PHE245	Hydrogen Bond Pi-Pi T-shaped	1.98 5.95
	ILE243	Amide-Pi Stacked	5.03
	ILE160	Pi-Sigma	3.88
kaempferol 3-O-robinobioside **Compound 4**	GLY161	Hydrogen Bond	3.07
	ILE160	Hydrogen Bond Pi-Alkyl	3.31 5.34
	CSX42	Hydrogen Bond	2.88
	TYR188	Hydrogen Bond Hydrogen Bond Pi-Alkyl	3.05 3.21 4.23
	PRO298	Hydrogen Bond	2.67
	TYR159	Pi-Alkyl	4.19
	CYS242	Hydrogen Bond	2.92
	ALA300	Pi-Alkyl	5.33
	LEU299	Pi-Sigma	3.31
kaempferol 3-(6G-malonylneohesperidoside) **Compound 5**	LYS213	Hydrogen Bond	3.07
	ILE243	Hydrogen Bond	2.62
	TYR159	Hydrogen Bond Pi-Alkyl	2.80 4.80
	CYS242	Hydrogen Bond	2.16
	TYR188	Hydrogen Bond Pi-Donor Pi-Alkyl	3.10 3.83 4.37
	SER326	Hydrogen Bond	2.21
	GLY158	Hydrogen Bond	3.51 3.56
	GLY244	Hydrogen Bond	2.73
	ILE297	Pi-Alkyl	5.28
	PRO298	Pi-Sigma	3.95

The similarity of interactions and bond types are the main reasons for the closeness of binding affinity and free energy between each ligand and control (Apocynin A), indicating the good antioxidant ability of the tested metabolite compounds. According to the previous studies, other ligands with different chemical structures have shown antioxidant activity due to the similarity of interactions and active sites with the current study [29,30]. For example, similar to the interacting residues in Figure 4 and Figure 5, Lountos et al. [30] showed that residues LYS213,

ILE243, TYR159, CYS242, TYR188, ILE160, and GLY158 of NADPH receptor interact with all ligand analogs which is consistent with our findings.

Conclusions

The Soxhlet method showed superiority over the maceration method, where there was a significant difference with a sig value (2 tailed): 0.000 (<0.05). LC-MS analysis showed that the macerated Moringa leaf extract had 101 secondary metabolite compounds, which showed that the total composition of the most effective flavonoid compound derivatives as antioxidants in the macerated extract was 70.9872% with five main compounds, namely 3-(((2S,3S,4R,5S,6R)-4,5-dihydroxy-3-(((2S,3S,4R,5R,6R)-3,4,5-trihydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)-6-((((2R,3R,4S,5R,6R)-3,4,5-trihydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)methyl)tetrahydro-2H-pyran-2-yl)oxy)-2-(3,4- dihydroxyphenyl)-5,7- dihydroxy-4H-chromen-4-one; luteolin-7-glucose; kaempferol 3-O-robinobioside; kaempferol 3-(6G-malonilneohesperidoside). There are 83 secondary metabolite compounds in the soxhlet extract which shows that the total composition of flavonoid compound derivatives is 75.60657% with the 5 highest compounds: quercetin-3-O-glucose; kaempferol 3- Neohesperidoside; kaempferol-3-O-(6"-malonylglucoside); kaempferol 3-O-robinobioside; and kaempferol 3-(6G-malonylneohesperidoside), which the total composition of the soxhletation method is higher. Supported by the antioxidant compound content test with DPPH, the macerated extract has an IC.50 value of 32.092 ppm, while the soxhlet extract has an IC.50 value of 14.328 ppm. In the same context, molecular docking of the main compounds identified on NADPH oxidase showed a higher binding affinity for Kaempferol 3-(6G-malonylneohesperidoside) (-10.1 kcal/mol), followed by Kaempferol 3-O-robinobioside and kaempferol 3-neohesperidoside (-9.6 kcal/mol), kaempferol-3-O-(6"-malonylglucoside) (-9.2 kcal/mol), 3-(((2S,3S,4R,5S,6R)-4,5-dihydroxy-3-(((2S,3S,4R,5R,6R)-3,4,5-trihydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)-6-((((2R,3R,4S,5R,6R)-3,4,5-trihydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)methyl)tetrahydro-2H- pyran-2-yl)oxy)-2-(3,4- dihydroxyphenyl)-5,7- dihydroxy-4H-chromen-4-one (-9.0 kcal/mol), luteolin-7-glucoside and quercetin-3-O-glucoside (-8.8 kcal/mol), and ternatin C3 (-6.9 kcal/mol), compared to the control (Apocynin A). These results prove the significant antioxidant capacity of the studied plants, which can be considered for further analysis in their use as possible antioxidant agents in the pharmaceutical industry.

Acknowledgement

The authors express their sincere gratitude to STIKES Karya Putra Bangsa Tulungagung for the internal research grant provided to support this study. Appreciation is also extended to Universitas Jambi, particularly to Indra Lasmana Tarigan, M.Sc., for conducting the *in silico* study. The authors further acknowledge Gambia University for the valuable research collaboration.

Author Contributions

A.L.K performed maceration and soxhlet extraction, A.M, S, and M.S.S were involved in LC-MS based secondary metabolite profile analysis, A.M. performed antioxidant assay, processed experimental data, performed analysis, drafted the manuscript, and designed figures. A.M. and T.W performed and evaluated In Silico Target Prediction. A.L.K helped in interpreting the results and working on the manuscript. All authors discussed the results and commented on the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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