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In silico approach of gambier extract for Diabetes Mellitus and multivariate optimization of ultrasound-assisted extraction rich polyphenol using full factorial design

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ABSTRACT: The gambier leaf from *Uncaria gambir* (W.Hunter) Roxb. is one of the promising natural agents as an antidiabetic candidate. Through an *in silico* approach, the study delves into the mechanisms of the extract's potential compounds, aiming to comprehend the molecular mechanisms underlying its antidiabetic activity. Additionally, an investigation was conducted to determine the most advantageous outcome of gambier leaf extract and the characteristics of the ideal extract. The extraction method used in the study was ultrasound-assisted extraction (UAE). Data analysis was done using a full factorial design 23 modeling approach. The extraction process involved three key factors: time, temperature, and material-solvent ratio. The observed responses included yield, total flavonoid content (TFC), and antioxidant activity (IC_{50}). Optimal results for the gambier leaf extract were achieved with a 30-minute extraction duration at 50°C using a material-solvent ratio of 1:10, which resulted in an extract yield value of 11.87%; total flavonoid content of 467.51 mgCE/g and antioxidant activity value (IC_{50}) of 66.01 µg/mL with a desirability value of 0.998. The optimal gambier leaf extract characteristics are a moisture content of 0.13% and a drying shrinkage of 0.15. The optimal extract has been demonstrated to contain phenolic compounds, flavonoids, and tannins. The validation results for the optimal extract condition obtained are 95% PI low and 95% PI high. Network pharmacology identified five compounds and three target proteins associated with gambier leaf in treating type 2 diabetes mellitus. Molecular docking analysis indicated that the interactions between SRC receptor and nicotiflorin, AKT1, and nicotiflorin, as well as TNF and procyanidin B2, have biological activity in treatment type 2 diabetes mellitus.

KEYWORDS: *Uncaria gambir* (W.Hunter) Roxb.; diabetes mellitus; ultrasound-assisted extraction; factorial design; network pharmacology; molecular docking.

1. INTRODUCTION

A persistent metabolic condition signed by elevated blood sugar levels and impaired carbohydrate, lipid, and protein metabolism is called type 2 diabetes mellitus. This condition can lead to several complications, leading to death [1]. Oral antidiabetic therapy, including biguanide, sulfonylurea, and *a-glucosidase* inhibitors, is administered to patients with diabetes mellitus (DM) to enhance the body's metabolism over an extended period [2]. However, prolonged use of these oral antidiabetic drugs can lead to

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side effects. This situation underscores the necessity for alternatives, such as using medicinal plants with antidiabetic properties, to minimize these adverse effects.

The efficacy of herbal remedies in effectively treating type 2 diabetes mellitus has been proven with numerous studies [3]. One promising natural agent as an antidiabetic candidate is the gambier plant *(Uncaria gambir* (W.Hunter) Roxb.). Research indicates that gambier plants lower blood sugar levels in animal models and exhibit potential enzyme inhibition *in vitro* assays, supporting this statement [4]. Utilizing the *in silico* approach is essential to advance previous research by discussing the mechanisms of individual compounds present in the extract and aiming to identify the compound components and understand their mechanisms in antidiabetic activity [5].

The composition of the compound content in gambier is highly dependent on the processing and treatment applied to gambier leaf [6]. It is essential to optimize the extraction of gambier leaf extract to obtain maximum bioactive and flavonoid compounds to maximize the crucial role of bioactive compounds in gambier plants. This optimization aims to enhance the utilization of gambier plants as a medicinal material with the potential for antioxidant properties.

The optimization process represents a normative approach for identifying the best solution in decision-making for a given problem. Optimization helps solve problems and achieve optimal results within specified constraints. It is conducted to minimize effort and maximize expected outcomes [7]. Design of Experimental (DoE) is a method suitable for the optimization process, focusing on optimizing the primary response from several variables. The goal is to optimize the response, and the DoE provides various design options with their respective functions, including factorial design. Factorial design is a specialized research design used to explore the effects of various conditions on research outcomes [8].

The choice of an extraction method is very important as it can affect the outcome and effectiveness of the extraction procedure [9]. The commonly employed extraction method is the conventional method. However, this method has several drawbacks, including a lengthy extraction time, the need for a large amount of solvent, and suboptimal extract results. Ultrasonic techniques can overcome the limitations of conventional extraction methods. The ultrasonic method utilizes acoustic waves with a 16–20 kHz frequency. It offers advantages such as accelerating the mass transfer rate [10], requiring a lower volume of solvent, shorter processing time [11], and reducing the extraction temperature [12]. Variables that could affect the extraction process comprise factors such as extraction temperature, solvent volume, amplitude size, ingredient ratio, solvent quantity, and duration of extraction time [13].

Network pharmacology involves constructing databases and networks to understand the development of diseases, analyzing these networks, validating findings through experiments, and offering potent tools to investigate the treatment mechanisms using natural substances and develop their bioactive compounds. Molecular docking is used to investigate the interactions between these active compounds and important therapeutic targets derived from network pharmacology. In exploring potential molecular mechanisms for treating type 2 diabetes, network pharmacology, and molecular docking-based approaches were used. Based on the considerations mentioned, research will be conducted on the *in silico* approach for *Uncaria gambir* (W.Hunter) Roxb. extract in curing type 2 diabetes mellitus and optimizing the extraction process using the ultrasonic method assisted by DoE. This research used time of extraction, temperature of extraction, and material: solvent ratio as independent variables. Responses related to extract yield, content of total flavonoids, and activity of antioxidant extracts will be analyzed through these variables.

2. RESULTS AND DISCUSSION

2.1. Extraction of Gambier Leaf

Standardization of the extract is conducted to guarantee the quality of the gambier leaf extract. This standardization involves a specific and a non-specific parameter. Specific parameters entail qualitative and quantitative chemical analyses of active compound levels linked to the extract's pharmacological activity [14]. The specific parameters observed in the standardization include organoleptic (colour, aroma, and shape) and extract yield. The standardized properties of gambier leaf extract include a dark brown color, a typical extract aroma, and a dense form. The percentage yield of gambier leaf extract is 11.87%. The yield shows the effectiveness and efficiency of the extraction method used. A higher yield value signifies a greater amount of extract produced. The requirement for high extract yield is that it should not fall below 10%.

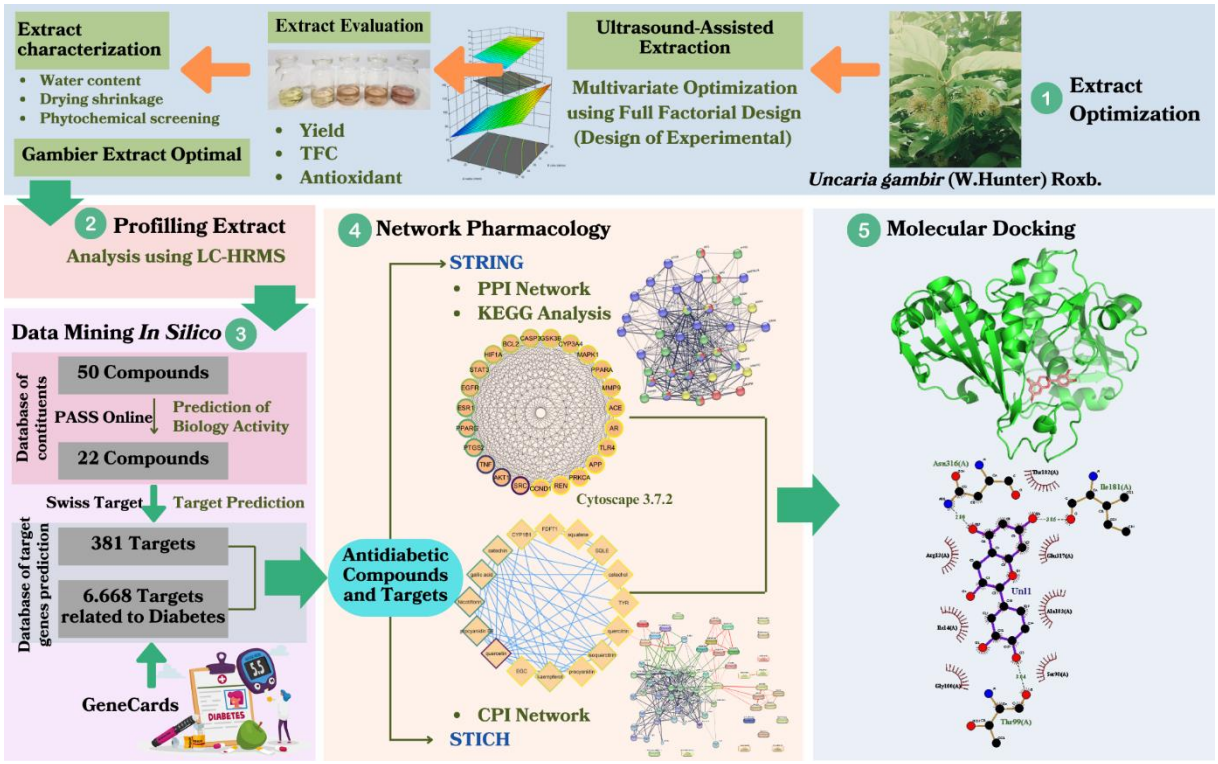

Figure 1. Flow chart of the multivariate optimization and *in silico* study using network pharmacology-docking.

2.2. Optimization of Gambier Leaf Extract with Full Factorial Design

Extract yield is the ratio between the mass of gambier leaf extract obtained and the mass of gambier leaves before extraction. Calculation of the amount of yield is used to determine the amount of secondary metabolites carried by the solvent. Still, the yield results cannot determine the type of compound the solvent carries [15]. The extraction results using ultrasound-assisted extraction with full factorial design (FFD) 2^3 are displayed in Table 1.

Table 1. Yields of percent yield, TFC, and antioxidant activity of extracts at each run.

Run	A: Time (min)	B: Temperature (°C)	C: Ratio (b/v)	R_1: Yield (%)	R_2: TFC (mg CE/g)	R_3: IC_{50} Antioxidant (µg/mL)
1	30	20	1:7	6.43 ± 0.12	450.57 ± 6.36	72.48 ± 1.75
2	10	50	1:7	5.70 ± 0.10	329.10 ± 0.49	101.06 ± 1.52
3	10	50	1:10	6.90 ± 0.10	322.03 ± 2.24	112.45 ± 0.62
4	10	20	1:7	5.10 ± 0.20	245.48 ± 4.18	134.80 ± 1.52
5	10	20	1:10	6.83 ± 0.23	277.40 ± 1.76	125.85 ± 1.18
6	30	50	1:10	8.50 ± 0.10	479.94 ± 2.13	64.47 ± 0.99
7	30	50	1:7	6.70 ± 0.20	465.82 ± 4.01	68.20 ± 1.09
8	30	20	1:10	7.63 ± 0.15	358.48 ± 3.69	79.42 ± 0.50

CE = catechin equivalents; TFC = total flavonoids content

As shown in Table 1, it is known that the highest extract yield was obtained from the treatment of 30 minutes of extraction time, 50°C extraction temperature, and 1:10 ratio of material to solvent, with a yield of 8.5 ± 0.10%. The lowest yield was obtained from the treatment of 10 minutes extraction time, 20°C extraction temperature, and a 1:7 ratio of material to solvent with a yield of 5.1 ± 0.20%. The treatment of extraction time influences the yield of the resulting extract. The longer the extraction time, the more excellent the opportunity for the material to come into contact with the solvent so that the resulting yield increases. The difference in yield results with the treatment of material: solvent ratio affects the yield produced. It is suspected that the higher the ratio of solvent used, the greater the pressure exerted. The plasmolysis that occurs is even more significant and causes the release of cell fluid to increase. Some research states that if the amount of solvent used is added, the contact of the material with the solvent as an extraction medium is also more significant and can maximize the extract yield [16].

Low extraction temperature in the extraction process will cause the content in gambier leaves to be incompletely extracted [17]. This is because at too low a temperature, the material has not been in contact with the solvent, and the extraction process cannot run perfectly. Similarly, the amount of solvent used: if the solvent is too little, then the pressure is also small, causing the cell fluid in the material not to come out maximally.

2.2.1. Half-normal plot analysis

The yield of gambier leaf extract was analyzed using factorial modelling. Factorial modelling contains regression equations that can provide a model that tells the relationship between responses that have been determined so that an optimum condition of each response variable is known. Figure 2 (1a-1c) is used to determine normally distributed data by paying attention to the factor points on the graph. Based on the half-normal plot graph, it can be seen that the points spread over the line, indicating the data is normally distributed. The half-normal plot graph shows that the time, temperature, and ratio factors are on the right. Figure 2 (1a) shows that increasing factors can increase the observed yield response. Figure 2 (1b) shows that flavonoid content is increasing while the factors are increasing. Figure 2 (1c) shows time and temperature factors are located to the left of the line, which means that increasing the time and temperature during the extraction process can reduce the antioxidant activity in the sample.

2.2.2. Predicted vs actual analysis

The factorial design analysis of the prediction versus the actual graph illustrating the closeness of the optimization prediction to the response obtained can be seen in Figure 2 (2a-2c). The prediction vs. actual graph is the relationship between predicted and experimental values. Points on the data that are nearly a straight line illustrate a well correlation between the predicted response and the experiment performed.The predicted vs actual graph is also used to help detect observations that are not well predicted by the model. This is known from the points on the graph, where the more parallel to the line, the more precise and accurate the data from the sample. In Figure 2 (2a), it can be seen that the points on the graph are aligned close to the line, which means there is a good correlation between response and yield extract. Figure 2 (2b) indicates that the response obtained correlates well with the predicted response of the total flavonoid content of gambier leaf extract. Figure 2 (2c) shows the antioxidant activity data has a good correlation between the prediction results and the research conducted.

2.2.3. Two-Factor interaction analysis

Two-factor interaction analysis was employed to assess how the interaction of two factors influences the outcome. The interaction that was analyzed is the temperature and time factors, with pH as the main factor. The effect of each temperature and time factor interaction on yield, flavonoids, and antioxidant responses is shown in Figure 2 (3a-3c). Figure 2 (3a) shows a graph of the relationship between the interaction of 2 factors on the value of redemption produced. There is an interaction of temperature and time factors on the yield response. This is indicated by the red line, which is a high-level temperature factor with time, has a slope towards the black line as a low-level temperature factor with time and forms an upward pattern. Figure 2 (3b) shows that the factors used in the extraction process interact with the total flavonoid content of gambier leaf extract. This can be seen from the interaction lines that overlap on the graph and form an upward pattern. The interaction graph of gambier leaf extract's antioxidant activity (Figure 2 (3c)) shows an attractive ratio between the length of extraction time used and the extraction temperature. This is indicated by the black line, which is the lower-level time-temperature factor with a slope that is not parallel to the high-level temperature factor with time in the form of a red line and forms a downward pattern.

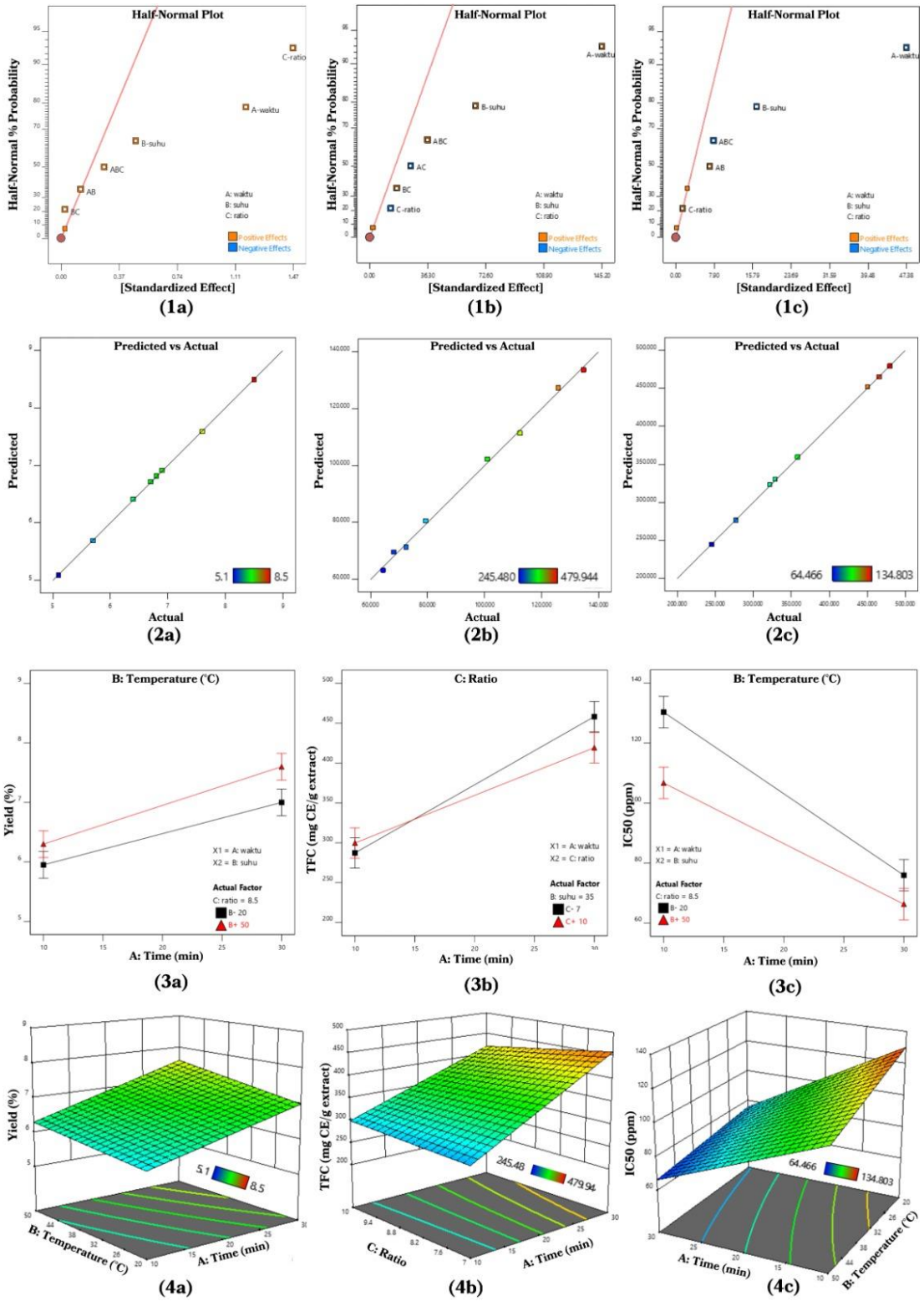

Figure 2. Results of full factorial design 2^3 analysis: (1) half-normal plot, (2) predicted vs actual, (3) two-factor interaction, (4) 3D Surface; (a) yield, (b) total flavonoids content, and (c) antioxidants.

2.2.4. 3D Surface analysis of actual factor time and temperature

3D Surface Analysis with actual time and temperature factors determine the effect of all factors on the response. The influence of each factor can be observed by changing the color gradations in the image. Areas with a reddish color show a higher response result, while increasingly blue areas show a lower response result. The influence of factors on yield response, flavonoids, and antioxidants is presented in Figure 2 (4a-4c). According to Figure 2(4a), it is shown that the yellow color has a higher response value than the green

and blue colors. This shows that the highest yield is yellow on the 3D surface, and the lowest yield is blue. Figure 2(4b) shows that run 6 indicates that it has the highest total flavonoid content.

Meanwhile, run 4 is in the blue position and has the lowest content. The color on the 3D surface obtained in this study, as seen in Figure 2(4c), indicates that run 6 with the most significant antioxidant activity (low IC_{50} value) is marked with blue on the 3D surface. Meanwhile, run 4 with low antioxidant activity (high IC_{50} value) is marked with an orange color on the 3D surface.

2.2.5. ANOVA and statistical parameters

The results obtained were then tested using analysis of variance (ANOVA) to determine each factor's significance level. Table 2 presents the outcomes of the ANOVA statistical analysis conducted on the parameters of yield value, total flavonoids, and antioxidant activity.

Table 2. Statistical analysis of gambier leaf extract from fitting model full factorial design 2^3

Response	SD	CV	R^2	Adj. R^2	Pred. R^2	Adeq. precision	Equation
R_1: Yield	0.03	0.52	0.9998	0.9989	0.9897	102.81	$R_1 = 6.71 + 0.59A + 0.24B + 0.74C + 0.06AB + 0.01BC$
R_2: Total Flavonoid Content	1.20	0.82	0.9998	0.9989	0.9897	83.66	$R_2 = 366.10 + 72.60A + 33.12B – 6.64C + 8.40BC – 12.85AC + 18.15ABC$
R_3: Antioxidants	2.44	2.57	0.9978	0.9921	0.9649	33.33	$R_3 = 94.83 – 23.83A – 8.31B + 0.71C + 3.50AB$

The ANOVA analysis of the percentage yield response reveals an R^2 value of 0.9998, indicating that the factors examined influence 99.98% of the resultant yield response. The remaining 0.02% is affected by other factors that are not taken into account within the model. The adjusted R^2 value in the table is 0.9989, while the predicted R^2 obtained is 0.9897. The validity of the fitted model is verified by comparing the values of the adjusted R^2 and the predicted R^2, as the difference between them is not more than 0.2. The model's adequacy is also considered good if the adequate precision value exceeds 4. The adequate precision value obtained in this modelling is 102.81, which means it has good modelling.

The ANOVA findings for the total flavonoid response indicated an R^2 value of 0.9998, falling within the standard range of 0 to 1 and approaching 1, suggesting that the statistical model is highly proportionate. Indicates that the factors under examination affect the response of total flavonoid levels by 99.98%. The remaining 0.02% is affected by other factors not considered within the model. The predicted R^2 value obtained was 0.9897, while the adjusted R^2 obtained was 0.9989. Based on this value, the predicted R^2 and adjusted R^2 values are appropriate because the difference in value is less than 0.2. The adequate precision obtained is 83.66, so the ANOVA statistical model can be used to organize modeling because it has an adequate precision value > 4.

The ANOVA results of antioxidant activity showed an R^2 value or multiple determination coefficient, which measures the suitability of the regression equation by showing the proportion or percentage of total variation in the variables. The R^2 value lies between 0-1, and the model is said to be proportional when it has a value close to 1. According to Table 2, it shows that the R^2 value is 0.9978. This implied that the factors studied influenced the antioxidant activity response by 99.78%, while the remaining 0.02% is affected by other factors that are not taken into account within the model. The adjusted R^2 value in the table is 0.9921, while the predicted R^2 is 0.9640. By paying attention to the adjusted R^2 and predicted R^2 values, it is evident that this model is suitable because the differences between the adjusted R^2 and predicted R^2 values do not exceed 0.2. The adequate precision result obtained in this modeling is 33.33 which means it has good modeling.

The results of each response (yield, total flavonoids, and antioxidant activity) were then tested for statistical parameters. This test consists of the percent contribution and p-value of each factor. This test aimed to ascertain the contribution and significance of each factor to the desired response. The equation derived from the results of the factorial design method, illustrating the effect of time of extraction (A), the temperature of extraction (B), and the ratio of material and solvent on extract yield (C), is provided in Table 2. Referring to the equation code for yield, it is observed that each factor used produces a positive response.

The positive value in the code equation indicates that increasing the factors used can increase the extract yield.

The positive value in the coded equation for total flavonoid content suggests that increasing the respective factor can enhance the total flavonoid content of an extract. Meanwhile, the negative coded equation value for the factor ratio suggests that this factor does not significantly influence the response of the total flavonoid content of the extract or may even reduce the observed response. Referring to the equation for antioxidant activity, it is observed that factor A, which is the time, and factor B, in the form of temperature used produces a negative response. The negative value in the equation indicates that decreasing the temperature and time factors can increase the IC_{50} value of the extract. This means that increased negative factors can reduce the activity of antioxidants from the gambier leaf extract used.

2.2.6. Determination of optimum conditions

The optimization of gambier leaf extract conditions involved analyzing factors such as time, temperature, and solvent-material composition on yield, total flavonoid content, and antioxidant response. This optimization process is needed to obtain the optimal solution from each research factor. The optimum conditions are determined to obtain the highest yield, total flavonoid content, and antioxidant activity with predetermined variable limits and as expected. The yield value obtained during the study was in the range of 5.1 - 8.5%. The yield response is at importance level 4. This is because the yield of the extract affects the content of total flavonoids and the activity of antioxidants.

The desired goal for the content of total flavonoid response is to achieve the highest possible level, thus the goal is set as maximization. Total flavonoid levels obtained during the study were in the range of 245.48 - 479.94 mgCE/g. The response of total flavonoid content is assigned importance level 5 because it strongly influences the activity of antioxidants in the extract. The expected goal of the extract's antioxidant activity response (IC_{50}) is minimized. This is due to the fact that a lower IC_{50} value indicates a higher antioxidant activity potential of a sample. Hence, the antioxidant activity response is assigned importance level 5.

2.3. Optimum Extract Characterization and Verification

2.3.1. Extract characterization

Characterization is carried out to assess the quality of extracts based on predetermined requirements. The moisture content in the extract is determined to maintain its quality and provide a range of water content. The results of determining moisture content in the extract is 0.13 ± 0.03%. This aims to avoid the rapid growth of mold in the extract [18]. Increased moisture content facilitates mold growth, potentially diminishing the biological activity of the extract during storage. The moisture content of the extract is contingent upon the duration and temperature applied during the drying process. As the temperature increases, the water content in the extract decreases. The principle of determining the water content is evaporating the water in the sample or heating it at 105°C for 5 hours [19].

Determining the drying shrinkage in extracts is imperative for standardizing medicinal plants, as it establishes a maximum limit or range for the amount of compounds lost during the drying process. This drying shrinkage test measures the residual substance after drying the sample in an oven at 105°C for 5 hours. Drying at 105°C will evaporate water, and compounds with a lower boiling point than water will also evaporate [20]. The results of determining drying shrinkage in gambier leaf extract is 0.15 ± 0,04%. Drying shrinkage meets the requirements that have been set, which is less than 10% [21].

2.3.2. Phytochemical screening

The optimum extract underwent phytochemical screening to the contents of phenolic secondary metabolites, flavonoids, tannins, saponins, and alkaloids. The phytochemical screening of flavonoids in gambier leaf extract showed positive (+) results marked by the formation of a red color after adding $MgCl_2$ reagent and concentrated HCl to the sample. The phytochemical screening of phenolic compounds showed positive results (+) indicated by the formation of a black color. Positive results in gambier leaf extract samples indicate phenolic compounds in gambier leaf extract; one of these compounds may be tannin, a polyphenolic compound. The other phytochemical screening of alkaloid compounds of gambier leaf extract showed negative results (-) because there was no orange precipitate after adding g drag-drop reagent to the sample. Testing of saponin content also showed negative results (-) because there was no foam on the sample.

2.3.3. Optimum extract verification

Verification of optimum conditions is carried out on the results of the optimal solution that the software has obtained. This needs to be done to see the accuracy of the selected program. The verification results that have been carried out are then compared with the results predicted by the program, which is presented in Table 3.

Table 3. Confirmation and verification of the optimum extract

Analysis	Predicted	Observed	SD	95% PI Low	95% PI High
Yield	8.48	11.87	0.03	7.87	9.11
TFC	478.88	467.51	2.99	426.75	531.01
IC_{50}	63.16	66.013	2.43	49.29	77.03

The prediction interval (PI) value for the extract yield response is 7.87 to 9.11, and in the observation results, the resulting extract yield is 8.87%, which is slightly lower than the lower limit of the PI value obtained. The total flavonoid content response observation results obtained at 467.51 mgCE/g are still within the PI range of 426.75 to 531.01 mgCE/g. While for the IC_{50} response, the PI is 49.25 to 77.03 µg/mL, which means that the observation results on the IC_{50} response are still in the prediction interval range.

2.4. Compound Profile Analysis of the Extract

2.4.1. Compound profile analysis using LC-HRMS

The results of testing gambier leaf extract samples using LC-HRMS obtained 50 compounds with the highest Area under curve (AUC) can be seen in Table 4. The high and low AUC shows the identified compounds' high and low concentrations. Those 50 compounds will be checked for their biological activity through PASS online.

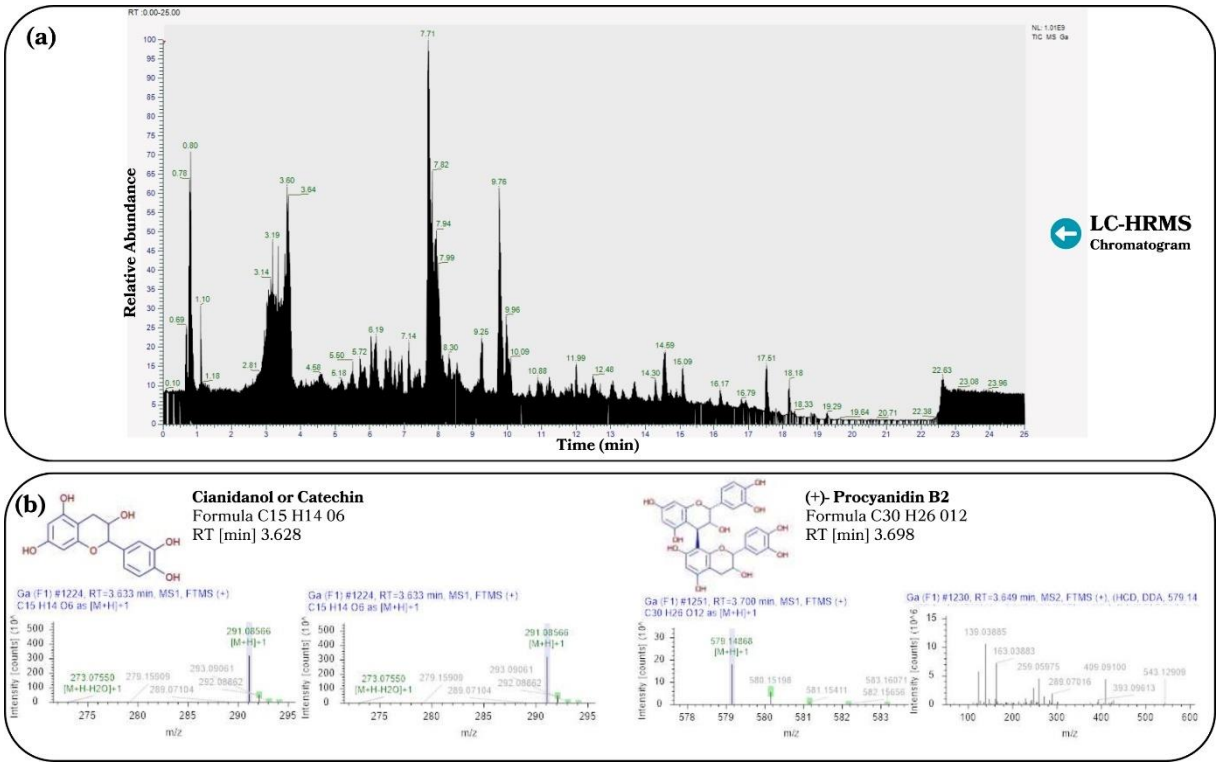

Figure 3. Profilling results of gambier leaf extract from *Uncaria gambir* (W.Hunter) Roxb. (a) LC-HRMS chromatogram and (b) Mass spectra of catechin and (+)- procyanidin B2.

Table 4. LC-HRMS result compounds with the highest max area of gambier extract

Peak	Compound Name	Molecular Formula	Molecular Weight	RT [min]	AUC (x10⁶)
1	Cianidanol	$C_{15}H_{14}O_6$	290.08	3.63	5156.81
2	N-(2-Methoxyethyl)-5-(2-methylphenyl) nicotinamide	$C_{21}H_{16}N_2O_2$	328.12	7.71	2687.70
3	Betaine	$C_5H_{11}NO_2$	117.08	0.80	564.90
4	3,4-Dihydroxybenzaldehyde	$C_7H_6O_3$	138.03	3.15	468.16
5	Ursolic acid	$C_{30}H_{48}O_3$	456.36	14.57	467.76
6	Hexyl 2-furoate	$C_{11}H_{16}O_3$	196.11	6.18	451.23
7	Epicatechin-(2beta->7,4beta->6)-catechin	$C_{30}H_{24}O_{12}$	576.13	6.61	444.81
8	5-Pentylresorcinol	$C_{11}H_{16}O_2$	180.11	9.26	431.23
9	(2E)-3-(4-{[(1R,2R)-1-Hydroxy-1-(4-hydroxy-3-methoxyphenyl)-3-{[2-(4-hydroxyphenyl) ethyl] amino}-3-oxo-2-propanyl] oxy}-3-methoxyphenyl)-N-[2-(4-hydroxyphenyl) ethyl] acrylamide	$C_{36}H_{38}N_2O_9$	550.42	7.14	336.17
10	(3R,6'R)-3',4',5',18'-Tetradehydro-5',6'- dihydro-beta, beta-caroten-3-ol	$C_{40}H_{54}O$	642.26	17.51	256.90
11	trans-caffeic acid	$C_9H_8O_4$	180.04	2.74	250.01
12	(3beta,4alpha,5alpha)-3-Hydroxy-4,14-dimethylcholest-8-en-7-one	$C_{29}H_{48}O_2$	428.36	18.17	240.42
13	Choline	$C_5H_{13}NO$	103.10	0.77	239.96
14	Adenine	$C_5H_5N_5$	135.05	0.81	210.01
15	Hyperoside	$C_{21}H_{20}O_{12}$	464.09	5.74	180.15
16	2-monolinolenin	$C_{21}H_{36}O_4$	352.26	11.99	140.32
17	2-Amino-1,3,4-octadecanetriol	$C_{18}H_{39}NO_3$	317.29	9.95	122.29
18	Gambiriin C	$C_{30}H_{26}O_{11}$	562.15	4.31	111.85
19	9-Oxo-10(E),12(E)-octadecadienoic acid	$C_{18}H_{30}O_3$	294.22	13.04	109.79
20	(1S,4S,5R,10S,13S,17S,19S,20R)-10-hydroxy -4,5,9,9,13,19,20-heptamethyl-24-oxahexacyclo	$C_{30}H_{46}O_3$	454.34	12.50	107.73
21	4,4-Dimethylcholesta-8,14,24-trien-3-ol	$C_{29}H_{46}O$	410.35	14.56	99.24
22	Tomentosic acid	$C_{30}H_{48}O_6$	504.34	10.07	98.71
23	Oleamide	$C_{18}H_{35}NO$	281.27	14.90	96.01
24	D-(+)-Proline	$C_5H_9NO_2$	115.06	0.80	94.30
25	Trifolin	$C_{21}H_{20}O_{11}$	448.10	6.13	91.09
26	(+)-Procyanidin B2	$C_{30}H_{26}O_{12}$	578.14	3.70	88.45
27	1-Aminocyclohexanecarboxylic acid	$C_7H_{13}NO_2$	143.09	0.81	88.31
28	N,N-Bis(2-hydroxyethyl)dodecanamide	$C_{16}H_{33}NO_3$	287.25	10.10	83.93
29	Quercetin	$C_{15}H_{10}O_7$	302.04	5.74	80.56
30	2-Furoic acid	$C_5H_4O_3$	112.01	0.82	79.64
31	Betulin	$C_{30}H_{50}O_2$	442.38	15.09	78.80
32	Rutin	$C_{27}H_{30}O_{16}$	610.15	5.52	78.65
33	(2,4,4-Trimethyl-2-pentanyl)benzene	$C_{14}H_{22}$	190.17	14.57	77.73
34	4-Hydroxybenzaldehyde	$C_7H_6O_2$	122.04	3.63	76.32
35	Gallic acid	$C_{21}H_{26}N_2O_3$	354.19	6.06	75.85
36	3-Oxolup-20(29)-en-28-oic acid	$C_{30}H_{46}O_3$	454.34	13.35	75.36
37	Di(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.28	17.55	71.48
38	1-Palmitoylphosphatidylcholine	$C_{24}H_{50}NO_7P$	495.33	12.52	61.63
39	12-Oxo phytodienoic acid	C18 H28 O3	292.20	12.56	60.09
40	Erucamide	$C_{22}H_{43}NO$	337.33	16.91	59.23

41	5,7-Dihydroxy-2-(4-hydroxyphenyl)-4-oxo-4H-chromen-3-yl 3-O-(6-deoxy-α-L-erythro-hexopyranosyl)-β-D-glycero-hexopyranoside	$C_{27}H_{30}O_{15}$	594.16	5.86	59.20
42	Nicotinamide	$C_6H_6N_2O$	122.05	1.10	56.61
43	Quercetin-3β-D-glucoside	$C_{21}H_{20}O_{12}$	464.09	5.81	55.35
44	D-(+)-Pipecolinic acid	$C_6H_{11}NO_2$	129.08	0.81	53.83
45	Quinine	$C_{20}H_{24}N_2O_2$	324.18	6.46	49.07
46	Nicotiflorine	$C_{15}H_{12}O_6$	288.06	3.04	48.07
47	Syringic acid	$C_9H_{10}O_5$	198.05	0.82	47.41
48	Methyl ibogamine-18-carboxylate	$C_{21}H_{26}N_2O_2$	338.20	7.56	47.08
49	4-Coumaric acid	$C_9H_8O_3$	164.05	3.62	46.15
50	2-(3-Hydroxy-4-methoxyphenyl)propanoic acid	$C_{10}H_{12}O_4$	196.07	4.60	43.24

2.5. Network Pharmacology

2.5.1. Activity prediction results of metabolites through PASS ONLINE

Each selected biological activity prediction has a Pa>0.3 value. Based on these predictions, 22 compounds with physical activities related to antidiabetic type 2 were obtained. The physical activities are antioxidant, antidiabetic, and antidiabetic type-2. The parameters shown in the PASS Online prediction results are Pa (Potential activity) and Pi (Potential Inhibitory). The compounds chosen for this study exhibit a predicted activity value of Pa>0.3, indicating that the compound is still in the active group having a certain bioactivity [24].

2.5.2. Compound target prediction results via SwissTargetPrediction

Potential Protein Targets from 22 gambier leaf extract compounds were entered into the SwissTargetPrediction database with a score of Pa>0.00. In total, 381 targets associated with gambier leaf compounds relevant to type-2 diabetes mellitus were identified.

2.5.3. Results of DM-2 target collection via GeneCards

A total of 6,668 integrated proteins that are thought to be therapeutic targets in type 2 diabetes mellitus, were obtained from searching the dialog box with the keyword "diabetes mellitus type 2" which has a score > 10.0.

2.5.4. Targeted collection results of gambier leaf in DM-2

The target proteins of gambier leaf compounds and those involved in treating type-2 diabetes mellitus were visualized using a Venn diagram to identify correlations or relationships between potential therapeutic targets of gambier leaf compounds and protein targets implicated in treating DM-2. From the Venn diagram results depicted in Figure 4 (a), a total of 340 correlated proteins were identified.

2.5.5. KEGG enrichment analysis

A total of 340 proteins that have been analyzed using STRING were then identified as burn wound healing signaling pathways through KEGG Pathway Figure 4 (b) and mapping of target proteins based on signaling pathways. There are 4 signaling pathways related to type 2 diabetes, namely Type 2 diabetes mellitus, insulin signalling pathway, adipocytokine signaling pathway, and apoptosis.

Type 2 diabetes is a metabolism disorder marked by insulin resistance, where the body's cells fail to respond appropriately to the insulin produced by the pancreas. All this leads to increasing blood glucose levels [25]. This condition is triggered through a combination of genetic and external factors and is characterized by elevated blood sugar levels coupled with insulin resistance, where cells lose sensitivity to insulin. Some factors, such as inflammation, oxidative stress, and mitochondrial disruption, lead to the progression of type 2 diabetes [26]. The insulin signaling pathway involves a series of biochemical events that facilitate glucose uptake by cells. AKT1 (Protein Kinase B) is activated by PI3K (Phosphoinositide 3-Kinase) and has an influential presence in managing multiple molecular processes, such as the stimulation of GLUT4 (glucose transporter type 4) translocation onto the surface of the cell, allowing glucose uptake by the cell [27].

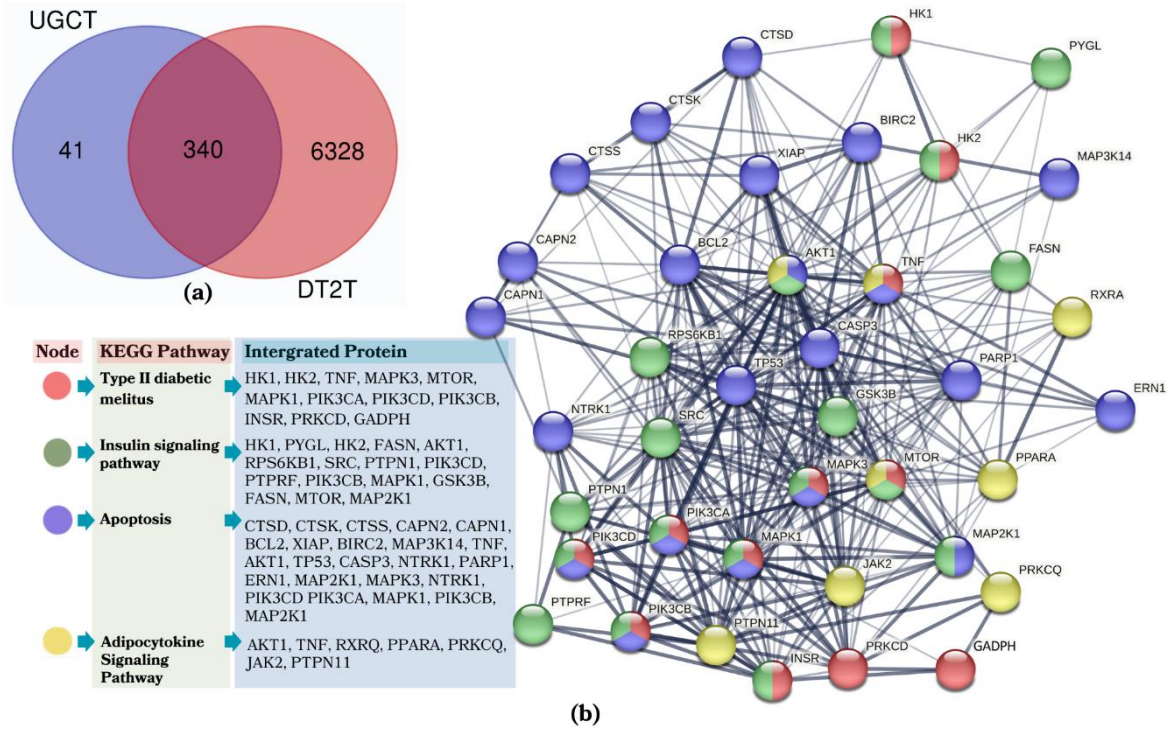

Figure 4. Network pharmacology analysis, (a) Intersection of target proteins in DM-2 and compounds in gambier leaf extract (b) KEGG enrichment on PPIs.

Fat cells, or adipocytes, have a prominent role in the regulation of metabolism and the development of insulin resistance. Adipocytes produce various adipocytokines, such as adiponectin and leptin, which are involved in regulating insulin sensitivity. In type 2 diabetes, alterations in adipocytokine signaling may affect insulin response and contribute to insulin resistance. Stimulation of adiponectin receptors initiates stimulation of AMP-activated protein kinase (AMPK), a primary regulator of diverse metabolic pathways, including increased insulin sensitivity [28, 29]. Apoptosis, also known as programmed cell death, may take place in pancreatic beta cells responsible for producing insulin. Increased beta-cell apoptosis can lead to decreased insulin production, exacerbating insulin resistance. Increased activity of apoptotic pathways in pancreatic beta cells can lead to beta cell death, reducing insulin production and exacerbating insulin resistance [30].

2.5.6. Antidiabetic compounds and targets

The provided data was uploaded in the STRING database, and Homo sapiens was the organism selected, in order to construct a protein interaction network. Subsequently, free nodes were eliminated, and they were visualized in Cytoscape version 3.7.2. The PPI network pharmacology visualization results are shown in Figure 5 (a), which consists of 23 protein nodes and proteins connected by 222 edges. The colour of the visualized circles varies according to the betweenness centrality value based on the mapping type in Cytoscape.

Table 6. The most important target proteins in type 2 diabetes mellitus

Protein Target	Betweenness Centrality	Degree	Closeness Centrality
SRC	0.0879	153	0.6384
TNF	0.0758	168	0.6595
AKT1	0.0779	175	0.6726

Among the analyzed proteins, three nodes exhibited centrality measures for both betweenness and closeness higher than the average. These proteins, tyrosine-protein kinase SRC (SRC), AKT serine/threonine kinase1 (AKT1), and tumor necrosis factor (TNF), were identified as the three most significant receptors associated with type 2 diabetes (Table 6). These proteins were then used as receptors in the molecular docking stage. The visualization result of CPI network pharmacology is shown in Figure 5 (b).

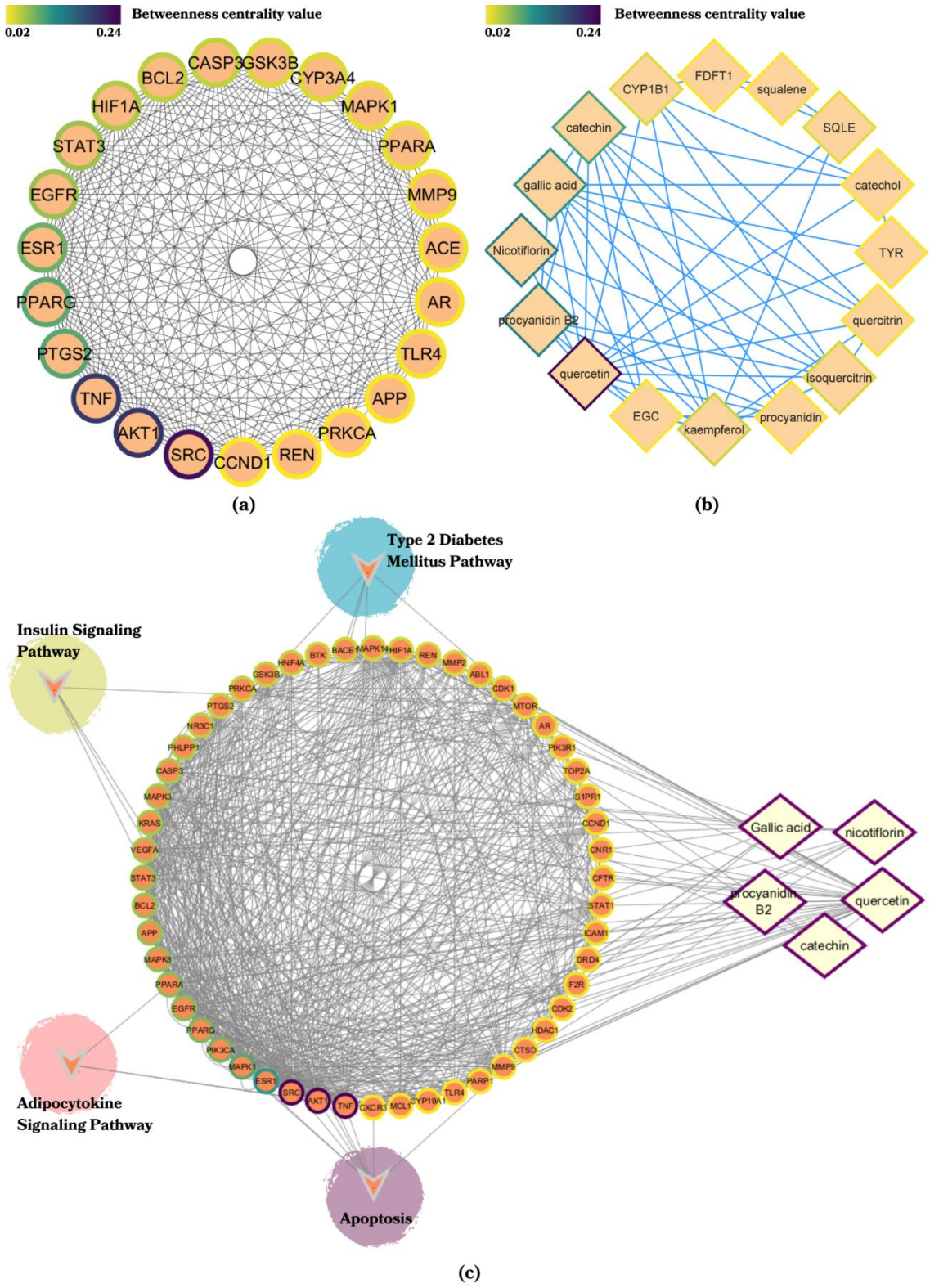

Figure 5. Visualization of network pharmacology gambier leaf compound against diabetes mellitus type 2 through cytoscape. (a) protein-protein interaction (PPI), (b) component-protein interaction (CPI), and (c) compound-target network of gambier extract constituents targeted to type 2 diabetes mellitus pathogenesis.

The molecular mechanisms involved in the relationship between the SRC receptor (Tyrosine-protein kinase SRC) and T2DM play a role in regulating various cellular signaling pathways, including the insulin pathway, which is key in regulating glucose metabolism [31]. SRC activity can modulate insulin receptor

(IR) activity [32]. SRC can interact with IR and inhibit insulin kinase activity. This may interfere with insulin signal transduction and its consequences, such as decreased cell response to insulin [33].

Tumor Necrosis Factor (TNF) is a cytokine with proinflammatory properties that participates in the body's inflammatory responses [34]. TNF may play a role in developing insulin resistance [35]. This cytokine has the ability to trigger signaling pathways that impede insulin responses in target tissues like muscles and adipocytes. A vital aspect of this signaling pathway, involving serine kinases, can disrupt the insulin receptor substrate (IRS) pathway, thus inhibiting insulin action [36]. The AKT1 (AKT Serine/Threonine Kinase 1) receptor regulates various cellular signaling pathways, including the insulin pathway. AKT1 activation generally occurs in response to insulin stimulation [37]. AKT1 also contributes to the inhibition of gluconeogenesis, which is the process of glucose formation from non-carbohydrate precursors. AKT1 activation inhibits the activity of enzymes involved in glucose production in the liver [38].

2.5.7. Compound-protein network

Visualization of compound-protein interaction (CPI) network pharmacology results through Cytoscape software using data imported from the STITCH database. Potential mechanisms of compounds were identified based on prediction analysis. Based on the analysis, an interaction network consisting of 16 nodes and 49 edges was obtained in Figure 5 (b). The purple color on the thicker border indicates stronger interactions in quercetin, procyanidin B2, nicotiflorin, gallic acid, and catechin. Quercetin is a flavonoid identified as a compound that may help reduce insulin resistance. Both laboratory and animal studies suggest that quercetin can improve insulin sensitivity and aid in the regulation of glucose metabolism [39]. Quercetin has an anti-inflammatory property that soothes inflammation within the body. Given that chronic inflammation is associated with the onset of T2DM, quercetin's capacity to mitigate inflammation might favor metabolic well-being [40-41]. Procyanidin B2 is a flavonoid group compound that can positively affect metabolic health and may contribute to managing T2DM. Procyanidin B2 might help to boost insulin sensitivity, minimize insulin resistance, and positively influence the metabolism of glucose [42]. Nicotiflorin, a flavonol glycoside compound has an anti-inflammatory property that soothes inflammation within the body. Since chronic inflammation is associated with insulin resistance and the onset of T2DM, nicotiflorin's ability to mitigate inflammation may have beneficial effects [43-44].

Gallic acid has been researched for its possible antidiabetic effects, acting as a potent antioxidant that may have the ability to regulate inflammation, apoptosis, and oxidative stress in various pathological conditions. Research has shown that gallic acid exhibits antihyperglycemic properties through its antioxidant and anti-inflammatory characteristics and its capacity to enhance insulin sensitivity and maintain glucose balance [48]. Catechin, a flavonoid present in tea and various plant-derived sources, has been explored for its potential as a therapy for diabetes. How catechins exert their antidiabetic effects include improving insulin sensitivity, reducing oxidative stress, regulating mitochondrial function, alleviating endoplasmic reticulum stress, demonstrating anti-inflammatory characteristics, lowering blood glucose levels, and impacting intestinal function [45].

2.6. Molecular Docking

The affinity value affects how stable the interaction between the ligand and the receptor is within the binding site region. A higher binding affinity value indicates weaker binding between the ligand and the receptor, while a lower binding affinity value signifies stronger binding between the ligand and the receptor [46-47]. The binding affinity values of the experimental compounds are provided in Table 7.

Table 7. Binding affinity value using auto dock vina

Protein	Binding Affinity (kcal/mol)				
	Quercetin	Procyanidin B2	Nicotiflorin	Gallic Acid	Catechin
SRC	-5.493	-5.993	-6.239	-3.972	-5.127
AKT1	-5.383	-5.880	-6.438	-4.593	-5.688
TNF	-5.226	-5.978	-5.733	-4.009	-4.880

The docking results of 3 test compounds show that the gallic acid compound that binds to the SRC receptor has the highest binding affinity value with the result of -3.972 kcal/mol, while the nicotiflorin compound that binds to the AKTI receptor has the lowest binding affinity value with a value of -6.438 kcal/mol. The more stable and robust a non-covalent interaction between the compound and the receptor, the result of the binding affinity value will be lower, indicating that the binding formed between the

compound and the receptor is strong and complex. This shows that the nicotiflorin compound is the test compound with the most potential as a receptor inhibitor in type 2 diabetes mellitus, with its specificity to the AKT1receptor. This is indicated by the lowest binding affinity value when compared to other compounds. Amino acid residues, which result from visualization of ligand interaction with the receptor, play an essential role in the area of the binding site [48]. Figure 6 illustrates the visualization outcomes of the tethering between the receptor and the ligand with the least binding affinity value.

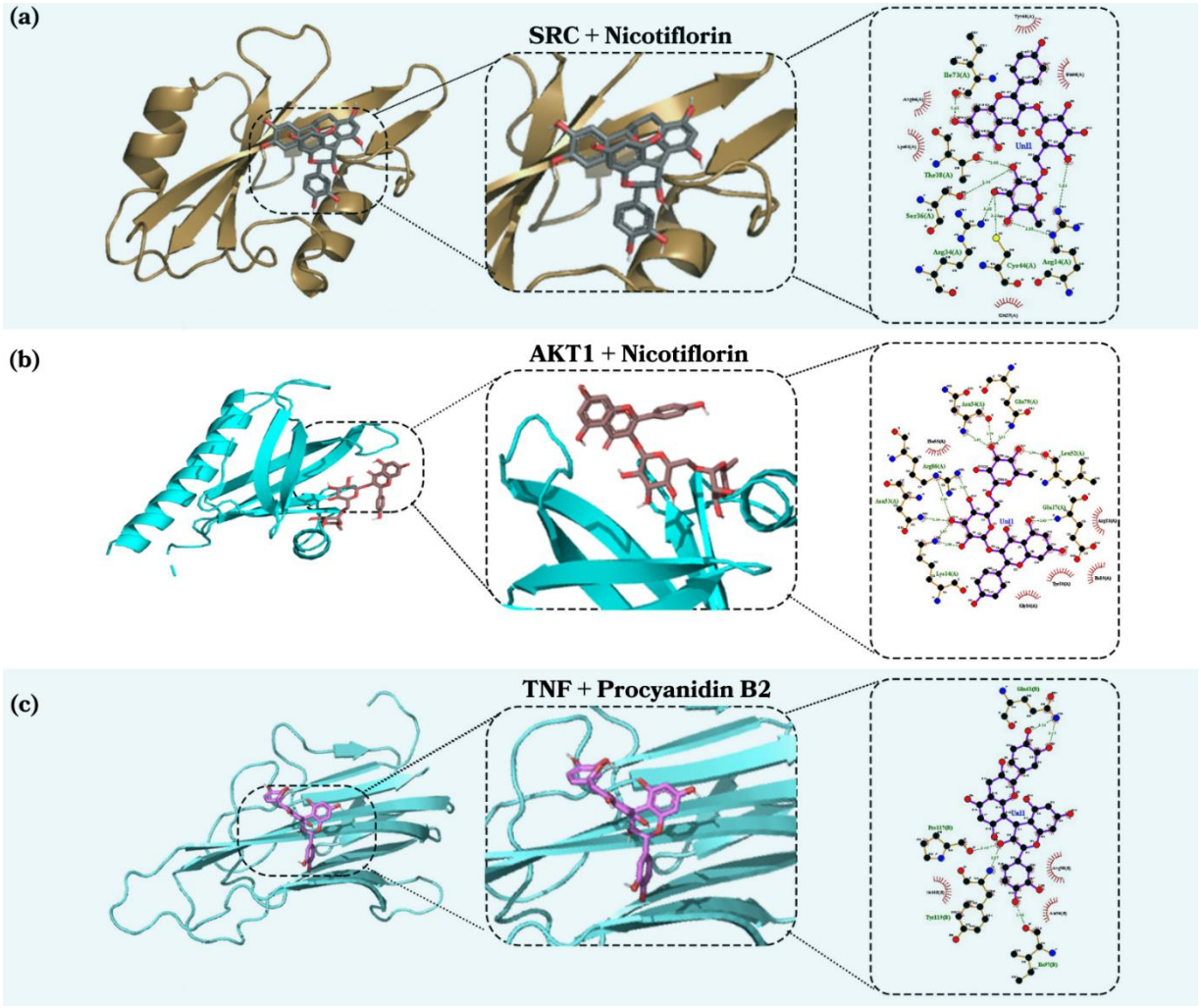

Figure 6. Visualization of molecular docking the receptor with the ligand and active site with the lowest binding affinity value result.

Figure 6 illustrates the interaction between the experimental ligand and receptors implicated in type 2 diabetes mellitus. The existence of amino acid residues bridging the ligand and the receptor proves their interaction. The interaction primarily involves hydrogen and hydrophobic bonds, which are crucial in determining the strength of the bond between the ligand and the receptor. The amount of hydrogen interactions between the compound and the amino acid residues produces a more vital interaction between the compound and the receptor [49]. From the analysis, it is evident that the interactions involving the SRC receptor with nicotiflorin, AKT1 with nicotiflorin, and TNF with procyanidin B2 exhibit biological activity in the treatment of T2DM.

3. CONCLUSION

The optimal conditions for extracting gambier leaf were achieved with a treatment duration of 30 minutes at 50°C with a material-to-solvent ratio of 1:10. This resulted in a yield of 11.87%, a total flavonoid content of 467.51 mgCE/g, and an antioxidant IC_{50} value of 66.01 mg/mL. The optimal characterization of

the gambier leaf extract includes a moisture content of 0.13% and a drying shrinkage rate of 0.15%, which indicates the presence of flavonoid, phenolic, and tannin compounds. SRC, TNF, and AKT1 are 3 proteins obtained from the analysis as the most responsible receptors in type 2 diabetes mellitus. Phytochemical compounds contained in gambier leaf extract potentially have activity as a cure for T2DM, namely catechin, quercetin, procyanidin B2, nicotiflorin, and gallic acid.

4. MATERIALS AND METHODS

4.1. Materials

The materials used, such as methanol, magnesium powder, concentrated HCL, NaOH, and $FeCl_3$, were purchased from Merck (Indonesia). Solvent ethanol of 96% and distilled water were obtained from Bratachem (Indonesia). Chemical standards in catechin and DPPH were purchased from Sigma-Aldrich (Singapore). Samples of gambier leaves were collected from plantations in Babat Toman Baru Village, Musi Banyuasin, South Sumatra. *Uncaria gambir* (W. Hunter) Roxb. was identified at the Herbarium Andalas University, Padang, West Sumatra, Indonesia, and authenticated with registration number 35/K-ID/ANDA/I/2023. The ligand used in this research is the active compound of gambier leaf extract, which has properties as an antidiabetic agent, which is drawn in the ".pdb" format and the SMILES code. The 3D structure of the receptor used for this research was downloaded on the Protein Data Bank (PDB) website.

4.2. Instrumentation

The tools used *In silico* analysis are hardware and software. The hardware used is an Asus Vivobook laptop (Intel Core i5 gen 8) with 8 GB RAM and 512 GB SSD. The software used is Cytoscape 3.8.2. The equipment used in this research was an oven (UN55-Memmert®), Ultrasound-assisted extraction equipment (GT-Sonic®), exhaust equipment (Winner-RQA®), UV-Vis spectrophotometer (Biobase®), GC-MS TRACE 1310 GC (Thermo Fisher Scientific, USA). LC-HRMS instrumentation was carried out using a Q-Exactive™ HF Hybrid Quadrupole-Orbitrap™ (Thermo Fisher Scientific, USA).

4.3. Sample Preparation and Extraction

Gambier plant samples were taken from Babat Toman Baru, Musi Banyuasin, Sumatera Selatan. The part of the gambier plant used is the gambier leaf. Fresh gambier plants were identified with identify number 35/K-ID/ANDA/I2023 at the Herbarium of Universitas Andalas, Department of Biology, Faculty of Mathematics and Natural Sciences, West Sumatera. Gambier leaves were washed thoroughly using running water and then oven-dried at 50°C for 24 hours. The dried gambier leaves were then pulverized and sieved until a homogeneous powder was obtained [50]. Gambier leaves were ground, and then extracted with ethyl acetate [51]. The sample was then put into a beaker glass covered with aluminum foil. The sample in the beaker glass is then inserted into the ultrasonicator tool according to each run treatment (Table 1). The extract was exhausted and stored in a dark bottle and sealed or packaged to avoid the entry of air from outside.

4.4. Design of Experimental using Full Factorial Design 2^3

The factors considered in this study consisted of 3 factors, namely: time (A: minute), temperature (B; °C) and material-solvent ratio (C; b/v). The three factors' upper and lower limit values were used in the design of the extraction formulation using ultrasound-assisted extraction (UAE). The time factor has an upper limit of 30 minutes and a lower limit of 10 minutes. The temperature factor has an upper limit of 50°C and a lower limit of 20 °C. Meanwhile, the material-solvent ratio factor has an upper limit of 10 and a lower limit of 7. The responses observed in this study consisted of three: yield (R_1), total flavonoid content (R_2) and antioxidant (R_3). The results of the combinations obtained based on the factors can be seen in Table 1.

4.5. Extract Yield, Determination of Total Flavonoid Content and DPPH assay

The yield of gambier leaf extract was obtained by comparing the weight of the extract with the weight of the gambier leaf powder, and multiplied by 100%[52]. Determination of total flavonoid content was measured by dissolving 0.005 grams of gambier leaf extract into 20 mL of ethanol, resulting in a concentration of 250 mg/mL. The solution was then diluted to 50 ppm. From the solution, 1 mL was pipetted, reacted with 0.3 mL of 5% $NaNO_2$ and 0.5 mL of 2% $AlCl_3$, incubated for 30 minutes, and added 0.25 mL of 2M NaOH. The sample was then measured absorbance with a UV-Vis spectrophotometer at a wavelength of 400-800 nm. The absorbance results were then substituted into the standard curve equation to

obtain flavonoid concentration (mgCE/g extract). DPPH radical scavenging assay was used to measure total antioxidant activity. The measurement was done by pipetting 0.2 mL of each sample into a bottle. DPPH solution was added to each sample as much as 3.8 mL. Close the bottle using aluminum foil, followed by incubation of the mixture for 30 minutes at room temperature in the dark. Next, using a UV-Vis spectrophotometer, the absorbance of the sample was measured at the maximum DPPH wavelength (400-800 nm).

4.6. Characterization Extract

The moisture content in gambier leaf extract was calculated by weighing 1 g of gambier leaf extract. Subsequently, the drying process was conducted in an oven at 105°C for a duration of 5 hours. Drying shrinkage is measured by 1 g gambier leaf extract heated in an oven (105°C; 30 minutes). The sample is then cooled in a desiccator with a closed state until it reaches room temperature, and then the fixed weight obtained is recorded [53].

4.7. Phytochemical Screening

The identification of saponins was carried out by adding 10 mL of hot distilled water and seeing the foam that formed. The identification of tannins and phenolics was carried out using water and $FeCl_3$ reagent, and colour changes were observed. Flavonoid identification is made by seeing changes in the color of the extract after adding water, $MgCl_2$ and HCl. Identification of alkaloids is done using Dragendroff reagent and will give a positive result if an orange precipitate is formed.

4.8. Compound Profile Analysis of Gambier Leaf Extract

4.8.1. Compound profile using LC-HRMS

The gambier extract underwent analysis utilizing Liquid Chromatography High-Resolution Mass Spectrometry (LC-HRMS) to profile untargeted metabolite. Untargeted metabolomics was conducted usinga Q-Exactive™ HF Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific, USA), combined with Vanquish Pumps (Dionex, Germany) and a PAL RSI autosampler (CTC Analytics AG, Switzerland). For chromatography separation, a Hypersil Gold aQ analytical column (50 × 2.1 mm, 5 µm; Thermo Fisher Scientific, USA) was selected with gradient elution of water and acetonitrile as a mobile phase. Heated electrospray ionization (HESI) was used in positive ionization mode, and Q-Exactive HF was operated in high-resolution sample scanning (1 m/z isolation window and 15,000 FWHM resolution for sample scanning) [58]. The results of LC-HRMS analysis in the form of non-volatile compounds that will be used as ligands are followed by computational methods to determine compounds that have the potential as therapeutic agents for type 2 diabetes [54].

4.9. Network Pharmacology

4.9.1. Activity prediction of metabolite compounds using PASS ONLINE

Prediction is done through the website (http://www.way2drug.com/passonline), by clicking "Go for Prediction" and selecting "Predict new compound" then "SMILE". Then enter the SMILE (Simplified Molecular Input Line Entry Specification) of the compound obtained from LC-HRMS and KNApSAcK database analysis. Selected compounds that have a predicted activity value of Pa>0.3 [55].

4.9.2. Prediction of metabolite compound target proteins using SwissTargetPrediction

Predictions were done through the Swiss Target database (http://*SwissTargetPrediction*prediction.ch/). Select the species "*Homo sapiens*" and enter the SMILE of each metabolite compound. Download the file in .xlsx format to obtain the target data of the compound and remove duplicate data [56].

4.9.3. Type 2 diabetes target protein collecting

The target disease was collected through the database (https://www.*GeneCards*.org/) and typed "type 2 diabetes" in the search box. Through the Venn diagram database (https://bioinfogp.cnb.csic.es/tools/venny/). Input the target compound of gambier leaf and the target of type 2 diabetes, then download the interaction results [57].

4.9.4. KEGG enrichment analysis

Enrichment was carried out through the database (https://www.genome.jp/kegg/pathway.html) to obtain the transduction signal analysis of each target protein of gambier leaf extract in type 2 diabetes [58].

4.9.5. Visualization of PPI and CPI using Cytoscape

Interactions between proteins (PPI) were analyzed through the website (https://STRING-db.org/), then select Multiple Proteins. Species were limited to the "Homo sapiens" group only and interaction score >0.4. Compound-protein interaction (CPI) was analyzed through the website (http://STITCH.embl.de/), then select multiple proteins. Species were limited to the "Homo sapiens" group only and interaction score >0.4. Visualization was carried out using the Cytoscape 3.8.2 application to analyze network topology using the network analysis function [59-60].

4.10. Molecular Docking

Targets exhibiting a high PPI score were selected for molecular docking to validate the connection between the active ingredients of the drug and their designated targets. The compounds' 2D structures were obtained from the PubChem database (http://pubchem.ncbi.nim.nih.gov/) and transformed into 3D structures using Chem 3D. These 3D structures were then acquired from the PDB (https://www.rcsb.Org/). The target protein was prepared by removing water molecules and small molecule ligands with the assistance of PyMOL. Subsequently, the protein was loaded into AutoDock-tools 1.5.6 for hydrogenation, charge distribution, and atom-type assignment tasks. Molecular docking was conducted utilizing AutoDock Vina 1.1.2, and the outcomes were further refined using PyMOL software to produce molecular docking diagrams. The docking outcomes were further analyzed in LigPlot+ to assess the precise binding interactions between the ligands and proteins.

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Author contributions: Concept – S.S.; Supervision and design – S.S., L.N., G.P.; Data collection and processing – A.A.M., S.H., N.A.F., I.S.; Literature research – S, G.P., J.T.W., P.I.; Materials and analysis – L.N., S, G.P.; Writing manuscript – A.A.M., S.S., G.P.; Critical review – H, S.H., J.T.W., P.I.
Conflict of interest statement: The authors declare no conflict of interest in the manuscript.

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