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Moringa oleifera as a potential bioactive agent against Gram-positive and negative bacteria: In-silico analysis of 1YN5 and 3RG1 receptor binding

CUT NILA THASMI[1,♥], HAFIZUDDIN HAFIZUDDIN[1], HUSNURRIZAL HUSNURRIZAL[1], DASRUL[1], AMALIA SUTRIANA[2], BASRI A. GANI[3], MUHAMMAD NAZAR[4]

[1]Laboratory of Reproduction, Faculty of Veterinary Medicine, Universitas Syiah Kuala. Jl. Teungku Hasan Krueng Kalee No. 4, Banda Aceh 23111, Aceh, Indonesia. Tel./fax.: +62-651-7551536, ♥email: cutnilathasmi@usk.ac.id

[2]Laboratory of Pharmacology, Faculty of Veterinary Medicine, Universitas Syiah Kuala. Jl. Teungku Hasan Krueng Kalee No. 4, Banda Aceh 23111, Aceh, Indonesia

[3]Department of Oral Biology, Faculty of Dentistry, Universitas Syiah Kuala. Jl. Syech Abdurrauf No. 4. Banda Aceh 23111, Aceh, Indonesia

[4]Department of Chemistry, Faculty of Education, Universitas Syiah Kuala. Jl. Teungku Hasan Krueng Kalee No. 4, Banda Aceh 23111, Aceh, Indonesia

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Abstract. *Thasmi CN, Hafizuddin H, Husnurrizal H, Dasrul, Sutriana A, Gani BA, Nazar M. 2024.* Moringa oleifera *as a potential bioactive agent against Gram-positive and negative bacteria: In-silico analysis of 1YN5 and 3RG1 receptor binding. Biodiversitas 25: 3411-3421.* The natural antioxidant extract of *Moringa oleifera* Lam. simultaneously possesses anti-inflammatory, anti-infertility, antimicrobial, and antioxidant properties. This research aims to investigate the active chemical compounds isolated from the leaves of *M. oleifera* and examine the chemical interaction between the selected compounds and the 1YN5 and 3RG1 proteins based on the molecular docking model. *M. oleifera* leaves were collected from the Kajhu Village, District of Aceh Besar, Province of Aceh, Indonesia. The bioactive compounds were isolated through cold extraction by using the maceration technique. Chemical analysis and characterization of bioactive compounds were carried out by Fourier Transform Infrared Spectroscopy (FTIR) and Gas Chromatography-Mass Spectrometry (GC-MS). The antioxidant bioactivity/Free Radical Scavenging Potency (FRSP) was determined using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical assay. The molecular docking process refers to the modified MOE software docking protocol. The data obtained were analyzed descriptively. The ethanolic extract of *M. oleifera* leaf contains 38 bioactive compounds. Several bioactive compounds have high peak area percentages, including Oxirane, hexadecyl- (20.22%), TRICYCLO [20.8.0.0E7,16] TRIACONTA (14.35%), acetic acid (CAS), ethyl acetate (11.32%), n-Hexadecanoic acid (8.94%), and oleic acid (8.12%). Molecular docking showed that oleic acid, oxirane, hexadecyl, and 1-Heptacosanol have greater affinity to the 1YN5 gene, while octadecanoic acid and-tetracontane have better binding affinity to the 3RG1 proteins. The bioactive compound 1-Heptacosanol bound both to the 1YN5 and 3RG1 proteins. The ethanolic extract of *M. oleifera* leaves possessed better IC50 (6.07 ppm), relatively twice lower when compared to the positive standard, Vitamin C (10.58 ppm). The *M. oleifera* ethanol extract exhibits antioxidant bioactivity in the very strong category. The bioactive compound 1-Heptacosanol shows binding affinity with both 1YN5 and 3RG1 proteins.

Keywords: Antioxidant properties, bioactive compounds, molecular docking, *Moringa oleifera*, 1YN5, 3RG1 proteins

INTRODUCTION

Endometritis, an inflammation of the endometrial layer of the uterus, is a reproductive health problem that is frequently ignored despite having significant consequences. The etiology of this condition is frequently associated with bacterial infection, with *Staphylococcus aureus* (*S. aureus*) identified as one of the key pathogens (Thasmi et al. 2021). An innovative therapeutic approach is required because the increasingly widespread antibiotic resistance frequently hampers the management of endometritis (Shafique et al. 2021).

Using natural products as therapeutic agents offers a promising avenue in this context. *Moringa oleifera* Lam. is known for its extensive pharmacological properties (Devi et al. 2020; van den Berg and Kuipers 2022; Ngemenya et al. 2024). Previous studies have demonstrated the effectiveness of *Moringa* extracts as potential candidates for inhibiting the growth of various pathogens (Pareek et al. 2023), including *Escherichia coli* and *S. aureus* (Unegbu et al. 2020). Bioactive compounds such as isothiocyanates in *M. oleifera* are believed to be important in their effective antibacterial properties (Jaja-Chimedza et al. 2017; Chak et al. 2020). However, the molecular mechanism behind its antibacterial activity, especially against *S. aureus*, still needs further exploration.

A study conducted by Rosmaidar et al. (2021) found that *M. oleifera* leaf extract has the ability to reduce the level of endometritis in Aceh cows. Setiawati et al. (2018) also reported that *M. oleifera* leaf extract reduced endometrial thickness significantly in Polycystic Ovarian Syndrome (PCOS) insulin-resistance model rats. *M. oleifera* leaf extract has been found to have an antibacterial effect against *S. aureus* and *E. coli* bacteria in vitro (Unegbu et al. 2020); both bacteria cause endometritis in cattle, resulting in losses and decreased livestock production.

The susceptibility to infections induced by Gram-negative bacteria is largely determined by innate immune responses

to the bacteria's cell wall Lipopolysaccharide (LPS) (Kim et al. 2023). The 3RG1 protein, which encodes the RP105 protein, has been identified as a key player in regulating innate immune responses to bacterial endotoxins, particularly LPS, a large, complex glycolipid found in the outer membranes of Gram-negative bacteria such as in *E. coli* (Ortiz-Suarez and Bond 2016).

Recent studies indicate that to prevent phagosomal destruction, *S. aureus* secretes a sequence of inhibitory proteins targeting bactericidal enzymes (de Jong et al. 2019). One family of these secreted inhibitors is Extracellular Adherence Protein (EAP) (Herdendorf and Geisbrecht 2019), especially the EapH2 protein (2.2 Å resolution, PDB code 1YN5) with high affinity as a selective inhibitor of Neutrophil Serine Proteases (NSP), including Cathepsin-G (C.G.) and Neutrophil Elastase (N.E.) (Mishra et al. 2023). The EAP of *S. aureus* mediates bacterial cell surface-extracellular host protein interactions as a secreted virulence factor (Herrera et al. 2019).

The immunomodulatory properties of specific compounds isolated from *M. oleifera* have come to attention. Possible mechanisms at play in this connection include those that regulate the activity of particular proteins, such as 3RG1, which is involved in immune response regulation (Vishwakarma et al. 2023). Extensive investigation into the potential modulation of 3RG1 activity and its subsequent influence on the immune system can be achieved by utilizing experimental and computational studies involving the active compounds of *M. oleifera*. The potential binding of active compounds derived from *M. oleifera* to the 1YN5 and 3RG1 proteins could be further elucidated through in-silico methods, including molecular simulation and docking. Therefore, this framework emphasizes the necessity for additional research that specifically investigates the consequences of *M. oleifera* compounds on the immune response and the regulation of the 1YN5 and 3RG1 proteins.

This study focuses on in silico analysis aimed to comprehend the binding affinities and inhibitory potential of bioactive compounds in *M. oleifera* towards these receptors. The research provides new insights into the mechanism of action of *M. oleifera* and its potential as a bioactive agent in the treatment of endometritis. Through the utilization of bioinformatics and molecular pharmacology methodologies, the purpose of this study is to establish a scientific foundation for the creation of novel treatment strategies that are not only effective but also safe for endometritis. The current challenges posed by antibiotic resistance are the focus of these strategies.

MATERIALS AND METHODS

Study area

This research used *M. oleifera* collected from the Kajhu Village, Baitussalam Sub-district, Aceh Besar District, Province of Aceh, Indonesia (Figure 1). *M. oleifera* leaf samples were carried to the Biology Laboratory of the Faculty of Mathematics and Natural Sciences, Department of Biology, Syiah Kuala University for identification and herbarium tests. The plant material (*M. oleifera*) was extracted from the Laboratory of Pharmacology, Faculty of Veterinary Medicine, Syiah Kuala University, Darussalam, Banda Aceh, Indonesia.

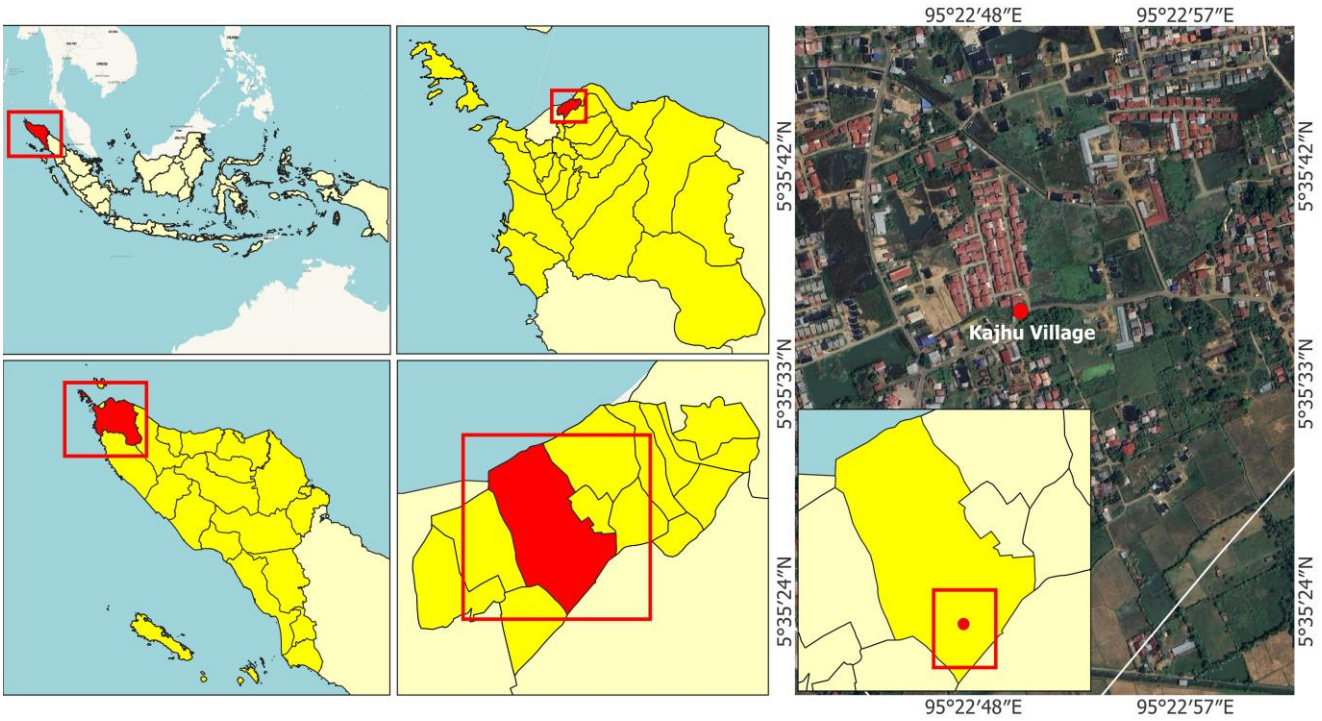

Figure 1. Location of *Moringa oleifera* leaf collection at Kajhu Village, Baitussalam Sub-district, Aceh Besar District, Province of Aceh, Indonesia (5°35'36.0" N 95°22'50.0" E)

Procedures

Preparation, extraction, and GC-MS analysis of Moringa oleifera

Furthermore, 1 kg of *M. oleifera* leaves were harvested and washed thoroughly using deionized water. The leaves were processed for two days of drying until wilted, then left for 48 hours in a 50°C oven. *M. oleifera* leaves were blended into a powder and stored airtight. The powder was soaked in 100 mL of 96% ethanol in a clean, flat-bottomed glass container. Changing the solvent was carried out for three days; the filtrate was concentrated with a rotary vacuum evaporator (Laboratory Rotary Evaporator BUCHI R-300 System, Swiss) at 50°C and 75 mmHg to obtain the concentrated extract (Gani et al. 2023a). The *M. oleifera* leaf ethanol extract was analyzed by GC-MS using a Shimadzu Japan QP2010PLUS equipped with a polymethyl silicon-coated fused G.C. column (2010). Conditions: 80-200°C for 5 minutes, then 200°C for 20 minutes. The Flame Ionization Detector (FID) temperature was 300°C, the injection temperature was 220°C, and the flow rate of the nitrogen carrier gas was 1 mL/min. 116.9 kPa. 30 m column, 0.25 mm diameter, flow rate of 50 mL/min (Yusuf et al. 2021; Gani et al. 2023b).

Fourier Transform Infrared (FTIR) analysis

The functional groups of chemical compounds in *M. oleifera* leaf extract were analyzed using Fourier Transform Infrared Spectroscopy (FTIR) (Shimadzu, 8400) at 4000-400 cm^{-1} and the transmittance spectrum. In the first step, the sample was placed on the surface of a transparent infrared prism whose refractive index was always higher than the sample. Then, the radiation beam was directed to one of the prism walls for the sample prism at an angle higher than the barrier. In this condition, perfect reflection occurs on the inner side of the prism. The reflected light exited through the second prism wall, where the light intensity and absorption spectrum were recorded (Gani et al. 2023a).

Molecular docking method

The molecular docking process refers to the modified MOE software docking protocol (Pagadala et al. 2017). The ligand (*M. oleifera* chemical compounds) was exposed to receptors (proteins) of Gram-positive (1YN5) (Mishra et al. 2023) and Gram-negative (3RG1) bacteria (Vishwakarma et al. 2023). The initial stage before molecular docking was geometry structure optimization and energy minimization of ligand and receptor by adding hydrogen atoms, partial charge setting using partial charge, and minimum energy conditioning using MMF94x force field. The solvation used is a gas phase with an RMS gradient of 0.001 kcal/mol using default and receptor output file in pdb format, while the ligand output file was mdb. Then, proceed to the docking stage, which begins with opening the optimized receptor file. Then docking was done by opening the "simulation-dock" window and setting the docking parameters: The placement method used was a triangle matcher with the number of rounds 1000x, the scoring function used was London dG, measurement (refinement) using refinement force field with the configuration of population repetition size of 1000 by the default MOE. The molecular docking process was ready when the system preparation had been completed. Evaluation of the docking results of the bond-free energy was seen in the output in mdb format. The ligand-enzyme complex selected is the complex that has the value of bond-free energy. Residue contacts and hydrogen bonds in the best ligand-enzyme complex docking results were identified and analyzed in three-dimensional media using ligPlot MOE software, then visualized in the ligand interaction program.

Antioxidant bioactivity of Moringa oleifera

The method of Shalaby et al. (2022) was utilized to determine the radical-scavenging effects of *M. oleifera* leaf extracts. A 2.0 mL aliquot of M. oleifera extract was added to a test tube containing a 0.16 mM DPPH solution (in methanol) for each concentration (2, 4, 6, 8, and 10 ppm), and prepare a vitamin C solution as a positive control with the same concentrations. Vortex each mixture for one minute and leave them at room temperature in the dark for 30 min. Measure the absorbance of each sample solution at a wavelength of 517 nm. Record the absorbance values for each concentration of M. oleifera extract and vitamin C. The percentage of scavenging was calculated using the following formula:

$$\text{Inhibition (\%)} = \left(\frac{(A_{Control} - A_{Sampel})}{A_{Control}}\right) x\ 100 \quad (1)$$

Where:

$A_{Control}$: Absorbance of DPPH

A_{Sample} : Absorbance of the sample

Data analysis

The data obtained were analyzed descriptively.

RESULTS AND DISCUSSION

FTIR analysis of *Moringa oleifera* leaf extract

Figure 2 shows the FT-IR profile of the *M. oleifera* leaf extract, which reflects 27 biomolecular peaks. Table 1 presents the phytocomponents, retention time, peak area percentage, and molecular weight of the *M. oleifera* extract.

Based on the FTIR results (Table 1), it showed that the *M. oleifera* leaf extract has a peak at wave numbers 3234.62, 3257.77, 3321.42, and 3346.5 cm^{-1} and showed the stretching vibration of the -NH_2 and -O.H. groups. The wave peaks of 2850.79 and 2920.23 cm^{-1} indicated the alkane functional group (C-H), and the wave peak of 1602.85 cm^{-1} showed the aromatic ring functional group. Wave numbers 1404.18; 1512.19 and 1539.20 cm^{-1} proved the absorption of the nitro aromatic (C-NO_2) functional group; the wave peak at 1361.74 cm^{-1} showed the absorption of the amine, amide (C-N) group, wave peak at 1029.99; 1062.78; 1103.28; 1180.44; and 1234.44 cm^{-1} showed the absorption of amine groups, amide alcohols, ethers, carboxylic acids, esters (C-O), while the wave peak at 518.85; 549.71; 594.08; 619.15; 638.44; 653.87; 682.8; 773.46; 813.96; 894.97; 920.05 cm^{-1} showed the absorption of halogen functional groups (Silverstein et al. 2005). Based on the analysis of the phenolic groups, it can be

predicted that the *M. oleifera* leaf extract contains phenolic or flavonoid compounds. The uniqueness of phenolic or flavonoid compounds with O-H groups characterized this.

GC-MS analysis of *Moringa oleifera* leaf ethanol extract

The compounds present in the *M. oleifera* leaf extract were identified through GC-MS analysis. Figure 3 depicts the GC-MS profile of the ethanol extract of *Moringa* leaves, which reveals 38 biomolecular peaks. The phytochemical components, retention time, percentage of wave crest area, and molecular weight are shown in Table 2. Table 2 and Figure 3 show the list of the chemical structures of the active components and their primary known applications, such as in pharmaceuticals, etc.

Table 1. The FTIR peak, intensity, and area of *Moringa oleifera* leaf extract (cm^{-1})

Peak	Intensity	Corr. intensity	Base (H)	Base (L)	Area	Corr. area
518.85	42.12	15.55	522.71	503.42	2.81	0.42
549.71	34.09	78.62	563.21	543.93	1.49	2.7
594.08	29.2	22.39	601.79	586.36	6.31	1.73
619.15	13.34	12.48	623.01	603.72	9.89	1.06
638.44	4.23	2.82	640.37	624.94	15.94	1.09
653.87	1.69	2.4	669.3	642.3	43.66	4.54
682.8	1.6	0.91	763.81	671.23	144.73	7.06
773.46	4.89	0.97	800.46	765.74	42.02	1.07
813.96	8.54	3.5	881.47	802.39	67.72	5.2
894.97	27.2	1.67	900.76	883.4	9.5	0.26
920.05	22.98	5.68	945.12	902.69	24.96	1.97
1029.99	1.57	5.24	1045.42	947.05	126.46	23.87
1062.78	1.67	2.57	1093.64	1047.35	71.23	8.18
1103.28	6.89	1.99	1165	1095.57	65.99	2.61
1180.44	18.88	1.96	1192.01	1166.93	17.66	0.57
1234.44	10.79	9.71	1319.31	1193.94	104.98	17.88
1361.74	12.82	2.67	1379.1	1321.24	47.54	2.22
1404.18	9.78	5.43	1440.83	1381.03	53.46	4.97
1512.19	23.61	5.98	1521.84	1485.19	19.64	1.45
1539.2	19.15	5.08	1548.84	1523.76	16.59	1.42
1602.85	8.37	35.08	1838.16	1550.77	132.75	65.55
2850.79	31.05	14.62	2873.94	2362.8	41.95	2.55
2920.23	17.34	29.58	2974.23	2875.86	50.87	18.64
3234.62	10.91	2.54	3250.05	2976.16	187.95	20.39
3257.77	11.04	0.09	3292.49	3251.98	38.49	0.1
3321.42	11.9	0.08	3338.78	3317.56	19.55	0.05
3346.5	12.13	1.7	3657.04	3340.71	166.92	23.21

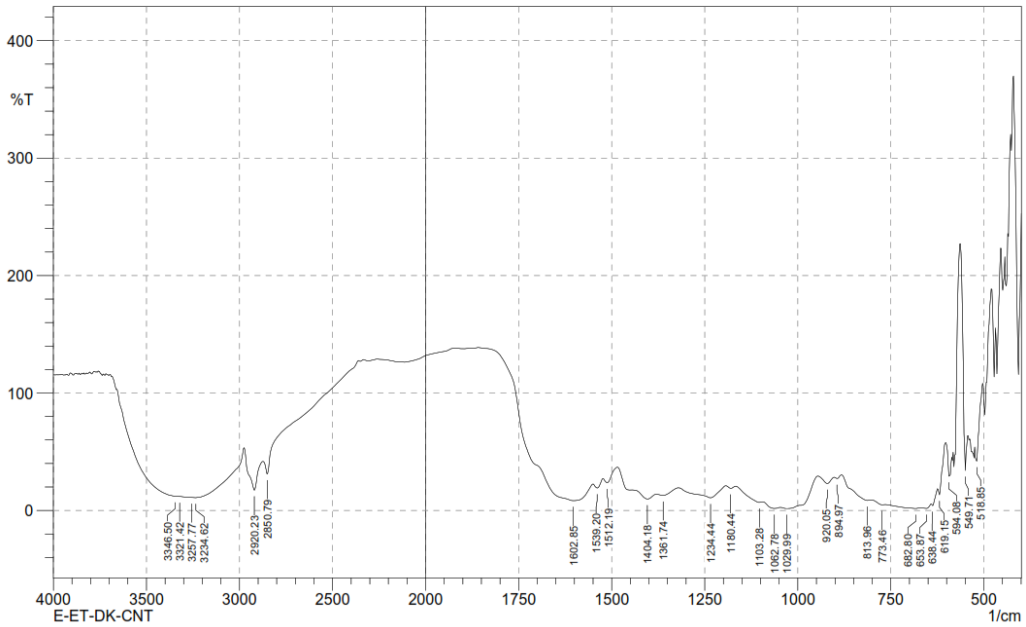

Figure 2. The FTIR spectrum of the *Moringa oleifera* leaf extract in the wavenumber region of 4000-500 cm^{-1}

Table 2. Phytochemical compounds in the *Moringa oleifera* leaf extract, retention time, and % peak area with molecular weight (g/mol)

Name	Formula	M.W. (g/mol)	R.T	Area	Area%	Bioactivity	Ref.
Oleic acid	$C_{18}H_{34}O_2$	282.4614	23.24	116071204	8.12	Antimicrobial, Anti-inflammatory, antitumor	Santa-María et al. (2023)
Octadecanoic acid	$C_{18}H_{36}O_2$	284.4772	23.38	40236010	2.81	Antimicrobial, lowers High Density Lipoprotein (HDL) cholesterol	Kavitha and Uduman (2017; van Rooijen et al. (2021)
Oxirane, hexadecyl-	$C_{18}H_{36}O$	268.4778	26.702	289152784	20.22	Antimicrobial	Musa et al. (2015)
Tetracontane	$C_{40}H_{82}$	563.0791	27.333	35357923	2.47	Antioxidant and antimicrobial	Swamy et al. (2017)
1-Heptacosanol	$C_{27}H_{56}O$	396.7329	32.719	6308096	0.44	Antibacterial, acetylcholin-esterase enzyme inhibitory activity	Koay et al. (2013)

Note: R.T: Retention Time; M.W.: Molecular Weight

Table 3. $\Delta G_{binding}$ value and affinity of five bioactive compounds of *Moringa oleifera* leaves extract with 1YN5 and 3RG1 proteins

Ligands	Receptor affinity (ΔG) (kcal/mol)			
	1YN5		3RG1	
	(ΔG)	Affinity	(ΔG)	Affinity
Oleic Acid	-9.31	Intermediate	6.78	Low
Octadecanoic acid	-6.61	Low	-9.54	Intermediate
Hexadecyl-oxirane	-9.49	Intermediate	-8.56	Intermediate
Tetracontane	-8.38	Intermediate	-8.48	Intermediate
1-Heptacosanol	-10.08	High	-8.1	Intermediate
Allantodapsone (Control for 1YN5 gene)	-6.91	Low	-	-
PGT (Control for 3RG1 gene)	-	-	-7.29	Intermediate

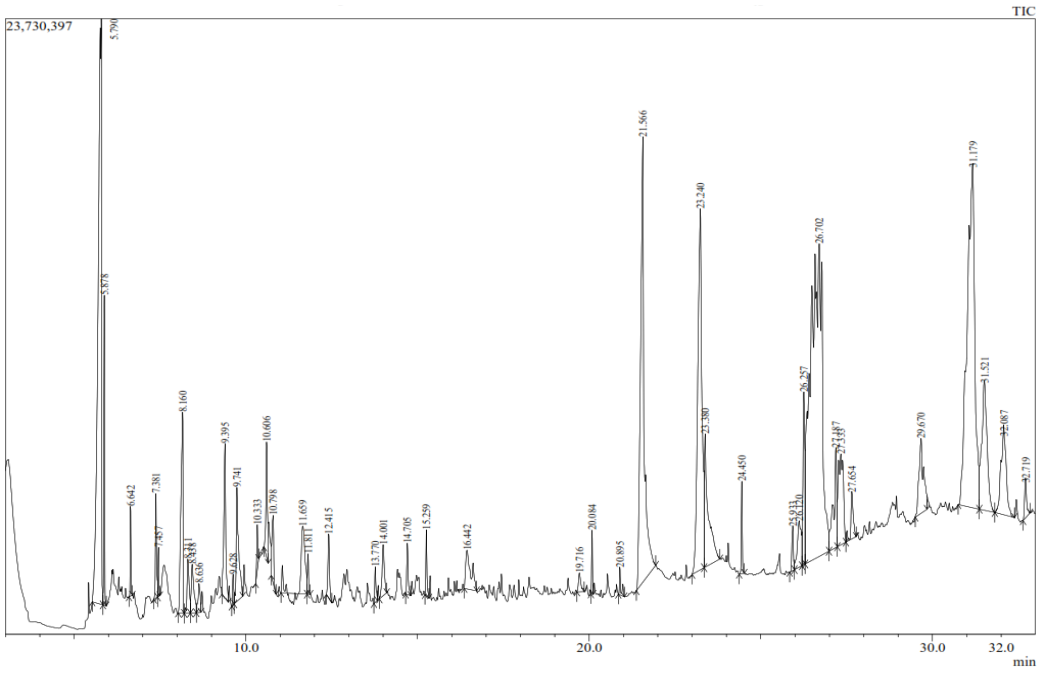

Figure 3. The GC-MS spectrum of the *Moringa oleifera* leaf extract. Peak started from the retention of 5.790 min to 32.719 min

Molecular docking of active compound of *Moringa oleifera* leaf extract

The result of binding affinity (ΔG_{bind}) of phytochemical compounds of *M. oleifera* leaf extract with 1YN5 and 3RG1 proteins is presented in Table 3. The results of ligand-receptor interactions are based on ΔG_{bind} values because each ligand binding to a protein macromolecule (receptor) produces a ligand conformation based on ΔG_{bind} rank. The smaller the ΔG_{bind} value, the more stable the ligand binds to the receptor. ΔG_{bind} is the energy the ligand requires when interacting or binding to the receptor binding site.

Figure 4 revealed the simulation results of ligand (*M. oleifera* chemical compounds) binding with receptors (proteins) of Gram-positive (1YN5) and Gram-negative (3RG1) bacteria with intermediate to high-affinity levels. Docking simulation used a reference control gene following the tested receptor to measure the effective binding affinity between the ligand and the receptor. Furthermore, the binding affinity value of the ligand with the receptor becomes a reference in simulations that work together to inhibit Gram-negative and Gram-positive bacteria bacterial infection.

In general, the ligands from the *M. oleifera* leaf extract that are most effective in binding affinity with the gene receptor (involved in the process of inhibiting bacterial infection) were oleic acid and hexadecyl-oxirane; according to Table 3, it is shown that bioactive compounds such as oleic acid and hexadecyl-oxirane, octadecanoic acid, tetracontane, and 1-Heptacosanol were predicted to be able to bind to the gene 1YN5 with an intermediate to high level of affinity. Oleic acid and hexadecyl-oxirane can act as inhibitors against the 1YN5 protein, which plays a role as a selective inhibitor of Extracellular Adherence Protein (EAP), especially EapH2 (2.2 Å resolution, PDB code 1YN5) (Herdendorf and Geisbrecht 2019) (Figure 4.A), however, it was predicted that they do not bind effectively to the 3RG1 protein. Bioactive compounds such as octadecanoic acid and tetracontane were predicted to be able to bind to the gene 3RG1 with an intermediate level of affinity (Figure 4.B). Meanwhile, the bioactive compound that binds to both 1YN5 and 3RG1 proteins was 1-Heptacosanol, with intermediate and high levels of affinity, respectively (Figures 4.A and 4.B). This indicates that the active compounds in the *M. oleifera* leaf extract, in silico, were assumed to be inhibitors of proteins 1YN5 and 3RG1, which can inhibit the process of bacterial attachment of *E. coli* and *S. aureus* to host cells.

The antioxidant bioactivity of *Moringa oleifera* leaf extract

Table 4 shows the results of the IC_{50} test for *M. oleifera* leaf extract. The IC_{50} for *M. oleifera* leaf extract was 6.07 ppm, so it has very strong antioxidant activity. Meanwhile, the comparative standard for vitamin C obtained a greater IC_{50} of 10.58 ppm, so it has robust antioxidant activity. This may be because the standard vitamin C had likely been oxidized, diminishing its capacity to donate protons to free radicals and producing a high IC_{50}. Vitamin C is a compound that is very easily oxidized due to the influence of temperature, light, and heat, so its ability to inhibit free radicals will also be reduced (Cresna et al. 2014). This study adheres to the Nikolić et al. (2014) value applied as the criteria for determining the measurement of the antioxidant activity.

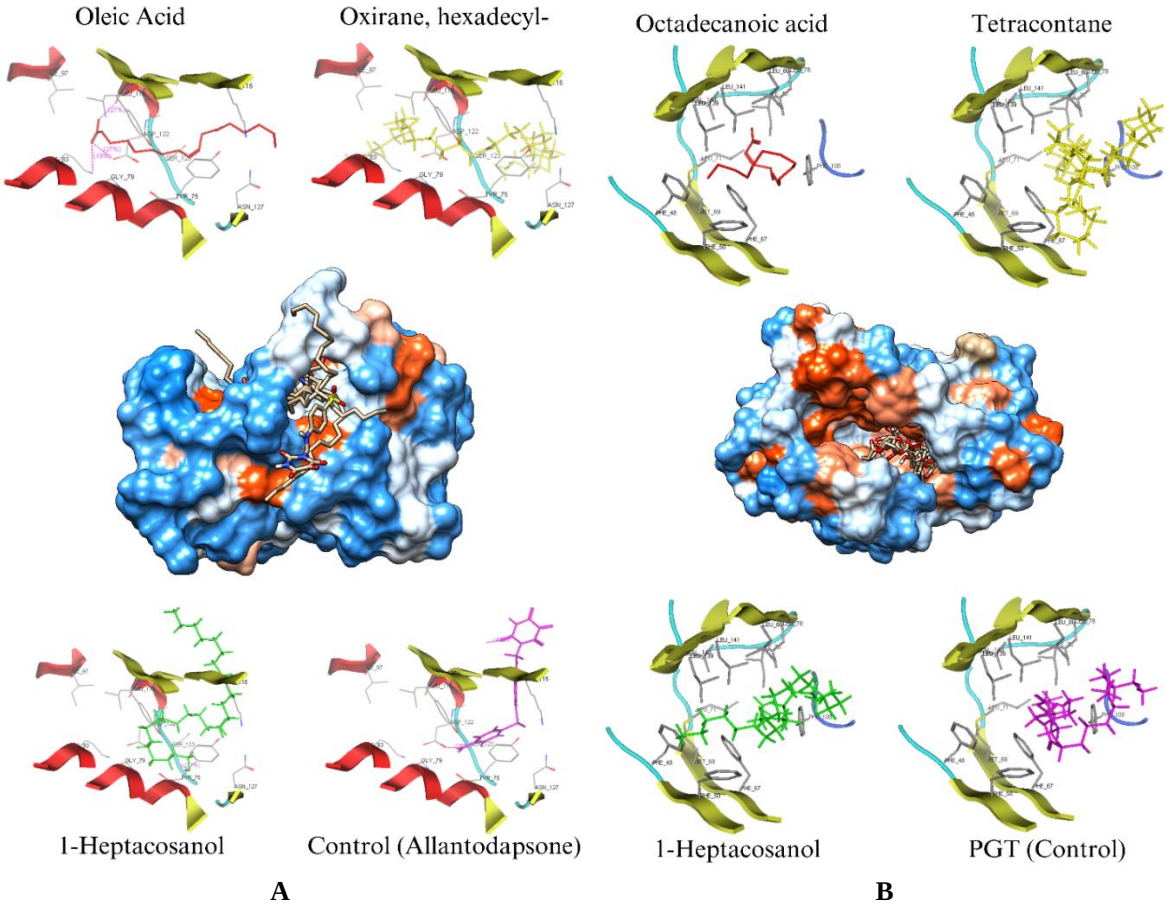

Figure 4. Visualization of docking results between the control ligand and three test ligands with the strongest affinity to the: A. 1YN5 protein; B. 3RG1 protein

Table 4. IC_{50} value of the *Moringa oleifera* leaf extract

Control (EtOH, Abs)	Cons. (ppm)	Absorbance		% Inhibisi		IC_{50}	
		Vitamin C	*M. oleifera*	Vitamin C	*M. oleifera*	Vitamin C	*M. oleifera*
0.108	2	0.075	0.099	30.56	8.33	10.58	6.07
	4	0.069	0.07	36.11	35.19		
	6	0.065	0.068	39.81	37.04		
	8	0.061	0.029	43.52	73.15		
	10	0.055	0.008	49.07	92.59		

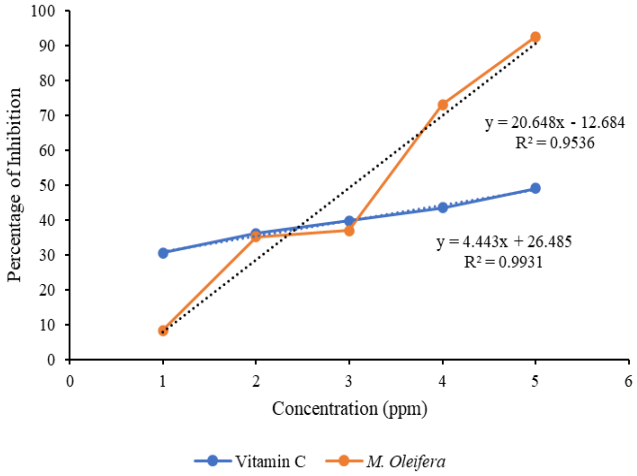

Figure 5. Regression correlation graph of inhibition percentage (IC_{50}) at various concentrations of the *Moringa oleifera* leaf extract

Figure 5 depicts the regression correlation of inhibition percentage (IC_{50}) at *M. oleifera* leaf extract concentrations. It was revealed that the higher the concentration of *M. oleifera* leaf extract used, the greater the inhibitory power of the resulting extract. The equation obtained on the relationship between maceration time and yield results is y = 20.648x-12.684 with R^2 = 0.9536. The regression analysis indicates that the correlation between the concentration of *Moringa oleifera* and Vitamin C with their inhibition percentages is extremely strong, with R^2 values of 0.9536 and 0.9931, respectively. However, the inhibition effect of *Moringa oleifera* increases significantly with concentration, exhibiting a stronger inhibition effect (20.648%) compared to Vitamin C (4.443%).

Discussion

FT-IR analysis of Moringa oleifera *leaf ethanol extract*

FTIR results of the *M. oleifera* leaf extract provided information about the functional properties of the *M. oleifera* leaf extract (Dutta 2017). The FTIR results of *M. oleifera* leaf extract proved that there are bending or stretching molecular vibrations. The presence of a wave peak (cm^{-1}) ranging from strong to moderate intensity (medium) was recorded wave at 3265.55, 2919.34, 1620.04, and 1537.73, 1022.74 and 617.96 cm^{-1} which was associated with the presence of hydroxyl groups (O.H.), carboxylic acids (RCOOH), Alkenes (-CH_2=CH_2), primary alcohols (-CH_2OH) and phenol compounds (Jeyakumar et al. 2020). Khalid et al. (2023) also reported that FTIR analysis of *M. oleifera* leaf extract identified the presence of major functional groups, including alcohols, alkanes, alkenes, nitro compounds, ethers, esters, carboxylic acids, aromatics, aliphatic bromo compounds, aryl disulfides, isocyanates, and phenols. The antioxidant and free radical scavenging ability of phenolic compounds such as phenolic acids and flavonoids mainly depends on the position and number of hydroxyl groups responsible for donating hydrogen in the aromatic ring structure (Devi et al. 2017).

The FTIR spectroscopy study conducted by Okechukwu et al. (2021) also found the presence of alcohol, alkane, aldehyde, carboxylic acid, aromatic and ester compounds observed from the methanol extract of *M. oleifera* leaves with wave peaks between 3500-3200 cm^{-1}, 3500-3100 cm^{-1}, 2750-250 cm^{-1}, and the wave peak of 900-675 cm^{-1} is a diagnostic marker for the presence of O.H., NH, C=O and C=C functional groups. The strain of the C-H group associated with H-C=O is usually slightly different in frequency due to the inductive effect of oxygen attached to the carbon and hydrogen atoms, thus weakening the bond. Therefore, a wide band in this spectrum region is diagnostic of the presence of O.H. groups. The results of FTIR analysis (1541.3-420.1 cm^{-1}) also revealed that *M. oleifera* leaf compounds can be aliphatic or aromatic.

Salimi et al. (2017) reported that the FTIR results of *Moringa* leaves produced absorption of clusters indicating the presence of flavonoids, namely at wave number 3429.14 cm^{-1} from O.H. absorption, 2936.08 cm^{-1} from aliphatic C-H absorption, 1713.04 cm^{-1} from C=O absorption, 1642.37 cm^{-1} from aromatic C=C phenol absorption, 1379.17 cm^{-1} from C-H absorption, 1239.14 cm^{-1} from C-O alcohol absorption, in C-H aromatic absorption there are numbers waves 957.36 cm^{-1}, 924.37 cm^{-1}, 884.75 cm^{-1}.

Therefore, the potential interaction between active compounds derived from *M. oleifera* and the 1YN5 and 3RG1 proteins is critical to understanding the molecular mechanisms involved. Molecular docking and simulation are examples of in silico techniques that can be used to forecast the binding affinity and mode of interaction between receptors and ligands (active compounds derived from *Moringa*) (1YN5 and 3RG1 proteins) (see Figures 4.A and 4.B).

Hydrogen bonds, including O.H. and NH_2 groups, are essential for molecular recognition (Bulusu and Desiraju 2020), possibly involving these groups. Phenolic compounds, which contain ligands bearing O-H groups, have the potential to establish hydrogen bonds with particular residues located in the binding site of receptors. The phenolic-

protein docking procedure comprises two fundamental stages: determining the binding affinity and predicting the conformation of the phenolic compound (ligand), including its position and orientation within the sites of the protein (receptor) (Meng et al. 2011; Shahidi and Dissanayaka 2023). Observing an aromatic ring within the *Moringa* extract suggests potential π-π interactions with the binding sites of 1YN5 and 3RG1. Zhu et al. (2021) reported that aromatic rings frequently participate in π-π stacking interactions, which may impact the binding of ligands to receptors.

C-N groups (Amine and amide groups) are nitrogen-containing organic compounds. These entities possess the ability to engage in hydrogen bonding and electrostatic interactions with other molecules, thereby potentially enhancing the molecule's overall binding affinity (Kumari et al. 2020). The C-O groups are compounds containing C-O groups that can potentially engage in hydrogen bonding and other interactions with the receptor sites (Horowitz and Trievel 2012).

Based on the findings, the FTIR analysis of the *Moringa* extract offers significant contributions to our understanding of its chemical composition. Moreover, the identified functional groups indicated possible molecular interactions that may play a role in the active compounds' binding to the 1YN5 and 3RG1 proteins. Additional research is required to verify these hypotheses and investigate the potential therapeutic ramifications of these interactions.

GC-MS analysis of Moringa oleifera *leaf ethanol extract*

Most of the compounds identified in the *M. oleifera* leaf extract have medicinal properties, some are commonly found in various medicinal plants. The presence of Pentetic acid compounds, 5-Octadecenal, Glucobrassicin, tetrapentacontane, 2-propenoic acid, pentadecyl ester, 3,4-dihydroxy mandelic acid is useful in therapeutic and pharmaceutical fields such as liver hydroxylation, analgesic antipyretic enzymes, rheumatism and has antimicrobial activity during phase I metabolism, inhibits uric acid production and inhibits arachidonic acid in the human body (Florence and Jeeva 2015). Hashim et al. (2017) reported the results of GC-MS analysis data on the ethanol extract of *M. oleifera* leaves, proving the presence of bioactive compounds, including flavonoids, tannins, and silicon compounds, in significant amounts.

Table 2 revealed five chemical compounds in *M. oleifera* leaf extract obtained from GCMS results that show higher wave peaks compared to other chemical compounds, including oxirane, hexadecyl (20.22%), tricyclo[20.8.0.0E7,16] Triaconta (14.35%), acetic acid (CAS), ethylic acid (11.32%), n-hexadecanoic acid (8.94%), and oleic acid (8.12%).

Acetic acid (CAS) ethylic acid is a colorless liquid with a pungent odor known as ethanoic acid. It is the second simplest carboxylic acid after formic acid (Kudo et al. 2023). In the food industry, ethanoic acid is commonly used as a preservative, flavoring agent, and pH regulator; it also produces several chemicals, such as vinyl acetate monomer, acetic anhydride, and esters (Trček et al. 2015). Regarding biological effects, it has been demonstrated that ethanoic acid has antimicrobial properties. It inhibits the growth of numerous bacteria and fungi, such as *E. coli*, *S. aureus*, and *Candida albicans* (Kudo et al. 2023). Another important function of ethanolic acid in plants is the induction of drought resistance. Acetic acid's novel mechanism of drought stress tolerance mediated by networks involving phytohormones, proteins, and chromatin regulation holds promising prospects for alleviating world hunger and halting desertification brought on by climate change (Kim et al. 2017; Wijaya et al. 2021).

N-hexadecanoic acid is a fatty acid that has anticancer activity (Sabithira and Udayakumar 2017), antitumor (Nabi et al. 2022), antimicrobial, antioxidant, antiatherosclerotic (Cho et al. 2010), and antiandrogenic (Komansilan et al. 2012). In addition, using an in-silico method, n-hexadecanoic acid has been used to design specific inhibitors of phospholipase A (2) compared with other inhibitors known as anti-inflammatory agents (Qureshi et al. 2016). Hexadecanoic acid and methyl esters, classified as fatty acids, are reported to have antibacterial, antioxidant, nematicidal, and insecticidal properties and reduce cholesterol (Nabi et al. 2022). Oleic acid is a major fatty acid found in *M. oleifera* leaves, with a concentration of 73.22% (Dhakad et al. 2019), also found in the leaves, flowers, immature pods, and seed oil of *M. oleifera* (Saini et al. 2016; Özcan 2020). Oleic acid has various biological effects, including anti-inflammatory activity, anticancer, antioxidant (Su et al. 2023), antimicrobial, skin health (Özcan 2020), cardiovascular health (Dhakad et al. 2019), and wound healing (Leone et al. 2016). The phenolic compounds of *M. oleifera* extracts are also associated with antimicrobial and antifungal activities (Milla et al. 2021).

Interaction of bioactive compounds of Moringa oleifera *with 1YN5 and 3RG1 proteins*

Molecular docking generates the binding energy between the ligand and protein, a crucial parameter providing information about the strength and binding affinity of the protein-ligand receptor complex. The lower the binding energy value, the higher the docking strength and the binding affinity (Salha et al. 2021). The present study involved molecular docking analysis to investigate the binding energies of bioactive compounds originating from *M. oleifera* with the selected 1YN5 and 3RG1 proteins. The binding energy value from molecular docking predicted the antibacterial potential of bioactive compounds from *M. oleifera*.

The molecular docking model confirmed that fatty acid compounds such as oleic acid, hexadecyl-oxirane, tetracontane, and 1-Heptacosanol bind effectively to the gene 1YN5 with an intermediate to a high level of affinity due to negative sign of Gibbs free energy, which confirms spontaneous process. Thermodynamically, the more negative the ΔG, the more potential the reaction or interaction between two molecules occurs (Sinurat et al. 2021). Organic acid compounds like oleic acid possess antibacterial agents due to the double bond and active sites of C=O and -O.H. chromophores (Agatonovic-Kustrin et al. 2023). Furthermore, the long chain-alcohol-like 1-heptacosanol showed high

binding affinity to the Gram-positive (1YN5) because the compound is an electron-rich species.

However, octadecanoic acid, a saturated fatty acid, appeared to bind moderately to the Gram-negative gene of 3RG1 but had a low binding affinity with the 1YN5 gene. The octadecanoic acid could bind to the gene through the carboxylic chromophore as the only potential binding site due to the phi electron provided by -COOH functional groups, specifically the carbonyl site (C=O) and the hydroxyl (-O.H.) (Kitazume et al. 2002), thus binding effectively with the 3RG1.

Oleic acid, functioning as a hydrophobic compound, has the ability to interact with hydrophobic residues on the EAP protein (Zeenat et al. 2023). Functional groups present in oleic acid, such as hydroxyl groups, have the potential to form hydrogen bonds with amino acid residues on proteins (Kusnandar 2019). Electrostatic interactions between the charges on oleic acid and the charges on protein residues may contribute to the binding process (Gheibi et al. 2020). Oleic acid has the potential to induce conformational changes in the EAP protein or affect its structural stability (Zeenat et al. 2023). The interaction mechanism can vary greatly depending on the compound's chemical nature and the protein's structure.

Previous research indicated that the RP105-MD-1 complex, where 3RG1 interacts with MD-1, is crucial in adjusting the immune response to LPS via the TLR4-MD-2 pathway. It has been demonstrated that the interaction between RP105 (3RG1) and MD-1 influences the specificity of the immune response to LPS, a major inducer of immune responses against Gram-negative bacteria. In addition to LPS recognition, the RP105-MD-1 complex and the 3RG1 gene may also regulate immune responses to various other pathogenic molecules (Ortiz-Suarez and Bond 2016). Further research into the TLR4-MD-2 pathway, which includes this complex, will provide more comprehensive insights into the mechanism by which 3RG1 modulates more extensive immune responses against pathogenic microorganisms.

The antioxidant bioactivity of Moringa oleifera *leaf*

Based on the report by Yuliani and Dienina (2015), it was explained that *Moringa* leaf infusion had antioxidant activity using the DPPH method with an IC_{50} of 2151.33 ppm obtained. This indicated that the infusion method by the IC_{50} value is high; thus, the antioxidant ability is relatively weak (>200 ppm) (Rahmayani et al. 2013). Riskianto et al. (2021)observed that the use of 70% ethanol in the extraction of *M.* leaves was able to provide DPPH antioxidant activity with an IC_{50} of 50.595 µg/mL, and quercetin compound as a standard was able to provide antioxidant activity with an IC_{50} of 0.538 µg/mL. Vitamin C could also be used as a standard, and based on research by Alim et al. (2021), it was reported that vitamin C possessed high antioxidants with an IC_{50} of 1.84 µg/mL. A compound is said to have very strong antioxidant activity if the IC_{50} value is <10 µg/mL, strong if the IC_{50} value is 10-50 µg/mL, medium if the IC_{50} value is 50-100 µg/mL, weak if the IC_{50} value is 100-250 µg/mL, and inactive if the IC_{50} value is >250 µg/mL (Nikolić et al. 2014).

The natural-derived phytochemical fingerprints occurring in *M. oleifera* leaves have been assumed to be associated with the various reported pharmacological activities, including estrogenic activities and myometrial contractility (Ajuogu et al. 2019). *M. oleifera* flowers are reported to have an oxytocic activity that can mediate endometrial contractility in buffalo (Devendra et al. 2010). In vitro administration of cold and hot aqueous extracts of *M. oleifera* significantly ($p<0.05$) increased uterine contractility with varying intensity. The contractile effect of cold *M. oleifera* aqueous extract (89.7%) was higher than that of hot *M. oleifera* (50.6%) (Attah et al. 2020).

M. oleifera has been reported to contain resveratrol (3,5,40-trihydroxy-trans-stilbene), a natural polyphenolic compound known for its rapid wound-healing characteristics (Patole et al. 2023). Resveratrol is reported to have potentially beneficial effects in a rat model with endometritis. Resveratrol reduced serum estradiol levels, increased serum progesterone levels, increased Estrogen Receptor (E.R.) expression in the uterine stroma, decreased ESR1 gene expression, and increased ESR2 gene expression. Administration of resveratrol combined with marbofloxacin was reported to increase E.R. expression in uterine glands and progesterone receptor expression in the uterine epithelium of model rats (Han et al. 2021).

Based on the results of binding energy between bioactive compounds from *M. oleifera* and proteins analyzed using molecular docking, it was found that compounds, including oleic acid, tetracontane, hexadecyl-oxirane, and 1-heptakosanol, have strong binding potential to the 1YN5 gene. This suggests that these compounds have antibacterial properties. These compounds potentially affect protein stability by interacting with it through hydrophobic and electrostatic interactions. It was concluded that the *M. oleifera* leaf extract contains 38 bioactive compounds. The bioactive compound 1-Heptacosanol shows binding affinity with the 1YN5 and 3RG1 proteins. The *M. oleifera* ethanol extract exhibits antioxidant bioactivity in the very strong category.

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