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Optimized Extraction of Kaempferol and Apigenin from *Moringa oleifera* Leaves and Their HIV-1 Reverse Transcriptase Inhibitory Activity

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Abstract: Kaempferol and apigenin, naturally occurring in Moringa oleifera leaves, have been suggested to inhibit HIV-1 reverse transcriptase (RT). However, their relatively low abundance in the plant matrix may limit the recovery and subsequent bioactivity evaluation. The study therefore aimed to optimize the extraction process to enhance the recovery of these bioactive flavonoids. A Box–Behnken design was applied to optimize defatting with n-hexane, microwave-assisted extraction (MAE) using 70% ethanol, and acid hydrolysis. The optimized conditions comprised n-hexane defatting, MAE with 70% ethanol for 17 min, followed by hydrolysis with 0.5 M HCl at 80 °C for 15 minutes. Under these conditions, the optimized extract exhibited a total flavonoid content (TFC) of 92.9 ± 0.5 mg quercetin equivalent (QE)/g extract, a total polyphenol content (TPC) of 165.7 ± 3.6 mg gallic acid equivalent (GAE)/g extract, with kaempferol and apigenin levels of 0.78 ± 0.01 and 0.47 ± 0.01 µg/g extract, respectively. The resulting value is twice the amount of TPC, kaempferol, and apigenin levels relative to the unoptimized extract, although the TFC content increased by 1.5 times. The optimized extract has an increased HIV-1 RT inhibitory effect, with an IC_{50} value of 223.13 µg/mL, compared to the untreated extract, which has an IC_{50} above 2000 µg/mL. It is suggested that the combination of defatting and acid hydrolysis significantly increased the levels of TFC, TPC, kaempferol, and apigenin in the extract, thereby enhancing its potential to inhibit HIV-1 reverse transcriptase.

Keywords: *Moringa oleifera*; HIV-1 RT; kaempferol; apigenin.

1. Introduction

Moringa oleifera, commonly called the "miracle tree," is native to India and widely distributed across Africa and Asia. As it is known for its rich nutritional profile and medicinal properties, *M. oleifera* has gained significant attention as a source of bioactive compounds with potential health benefits. The leaves of this plant contain a high concentration of essential vitamins, minerals, and bioactive compounds, making it a staple in traditional medicine

systems [1]. In South Africa, it has been traditionally used in managing Human Immunodeficiency Virus (HIV) infection, especially given the high prevalence of HIV/AIDS in the region [2].

Controlling HIV relies heavily on the effectiveness of antiretroviral (ARV) therapies, which target various stages of the virus's life cycle to prevent replication. HIV-1 Reverse Transcriptase (RT) is a crucial enzyme that plays a pivotal role in the early stages of HIV infection [1,3,4]. Despite the success of ARV therapy in reducing HIV infection, there is a need for additional efforts to mitigate the toxicity of existing drugs or discover new treatments that do not foster the emergence of resistant variants.

Among promising alternatives, kaempferol and apigenin—two flavonoids found in M. oleifera leaves—have shown potential to inhibit HIV-1 RT, suggesting a complementary approach to current ARV therapies [5]. Kaempferol and apigenin are flavonoids, a class of polyphenols commonly found in plant leaves and often present in glycoside forms. In *M. oleifera*, these compounds exist as apigenin-7-*O*-glucoside, kaempferol 3-*O*-glucoside, and kaempferol-3-*O*-rhamnoside, though in relatively low concentrations [6]. Optimizing extraction techniques to yield higher concentrations of these flavonoids could enhance their biological efficacy. Advanced methods, such as ultrasonic-assisted extraction (UAE) [7,8] and microwave-assisted extraction (MAE) [9], could effectively increase the concentration of these active compounds, potentially amplifying the anti-HIV activity of the extract.

This study aims to optimize the extraction technique to increase the level of kaempferol and apigenin in the crude extract of moringa leaves using microwaves, defatting, and hydrolysis. This study may guide the production of a kaempferol- and apigenin-rich extract that could inhibit HIV replication.

2. Materials and Methods

2.1. Materials.

M. oleifera leaves from Tawangmangu, Karanganyar, Indonesia, ethanol (Merck, Germany), methanol for analysis (Merck, Germany), methanol for HPLC (Merck, Germany), n-hexane, HCl, gallic acid (Markherb, Indonesia), quercetin (Markherb, Indonesia), kaempferol (Markherb, Indonesia), apigenin (Markherb, Indonesia), aquadest, PBS, HIV-1 Reverse Transcriptase assay kit (Mybiosource, USA).

2.2. Defatting with non-polar solvent.

Moringa leaf powder was defatted by adding the non-polar solvent n-hexane in a 1:5 ratio. The mixture was left to stand overnight. Afterward, the suspension was filtered, and the residue powder was dried in open air before use in the MAE process.

2.3. Microwave-assisted extraction (MAE).

Microwave-assisted extraction (MAE) was performed using a microwave system and a suitable glass apparatus comprising a round flask and a condenser. 10 g of leaf powder was placed in an Erlenmeyer flask and mixed with 70% ethanol solvent at a solid-to-solvent ratio of 1:25. The mixture was heated in a microwave at 240 W for 20 minutes. The mixture was filtered and evaporated using a rotary vacuum evaporator at 40°C. Subsequently, the yield was

calculated, the moisture content measured, and the thick extract was stored in light-protected vials at 4 °C until further processing [9].

2.4. Hydrolysis.

Two grams of thick moringa leaf extract were added to 50 mL of HCl in methanol (1:25 ratio). The mixture was heated at 80°C under reflux, with hydrolysis optimization by varying HCl concentrations (0.2, 0.6, and 0.9 M) and hydrolysis times (15, 30, and 45 minutes). After hydrolysis, the mixture was evaporated using a water bath and analyzed for total polyphenols (TPC) and total flavonoids (TFC) of the extract. This hydrolysis procedure followed established methods with modifications as necessary [10].

2.5. Response surface method (RSM) optimization.

The response surface method (RSM) with the trial edition of Design-Expert (Statease Inc., Minneapolis, MN, USA) was employed to optimize the extraction parameters for moringa leaves. This procedure utilized a Box-Behnken design with three components and three levels, yielding 15 extraction conditions. The measured responses were TFC (mg QE/g extract) and TPC (mg GAE/g extract).

2.6. Reverse-phase HPLC analysis.

Determination of kaempferol and apigenin levels in moringa leaf extract was done using the reversed-phase HPLC (Shimadzu) method with a C18 plus column (Zorbax, Agilent Technologies) as the stationary phase and a mixture of methanol (A) and 0.2% formic acid in water (B) in a 50:50 ratio with isocratic elution. The analysis was conducted at a wavelength of 365 nm using a UV-Vis detector, with a flow rate of 1 mL/min and an injection volume of 20 µL. The method's performance was evaluated by examining the linear range, limit of detection (LOD), limit of quantification (LOQ), repeatability, precision (%RSD), and recovery. These assessments were conducted in accordance with the ICH guideline Q2 (R2) on validation of analytical procedures.

2.7. Inhibitory activity of HIV-1 RT enzyme assay.

HIV-1 RT inhibition was evaluated using a kit procedure, with controls set at 100% inhibition (solvent without enzyme) and 0% inhibition (enzyme without inhibitor). ABTS substrate solution alone served as a blank control. Each treatment was performed in triplicate. Absorbance was measured at 405 nm, followed by percentage inhibition and IC_{50} value calculations.

3. Results and Discussion

3.1. Response surface method (RSM) optimization.

The extraction optimization using the Box-Behnken design in RSM was conducted to achieve optimal conditions for maximal or minimal responses aligned with the research objectives. Table 1 describes the Box-Behnken experimental design and response values for total flavonoid content and total phenolic content. The statistical model test indicated that the analysis results for both responses satisfied the established standards, with projected R^2 and modified R^2 values below 0.2 and an Adeq Precision score exceeding 4. The statistical analysis

results indicate that the model can predict optimal extraction conditions. The relationship between factors and responses can be observed in the 3D curves generated by the RSM system using the Box-Behnken method (Figure 1). These three-dimensional surface plots visualize the relationship between two independent variables (X and Y axes) and one dependent variable (Z axis). The contour shape of the response surface varies with the combination of response values from the X and Y variables [11].

Table 1. Box-Behnken experimental design and response values for total flavonoid content and total phenolic content.

Run	Factor			Responses	
	Extraction time (minutes)	Hydrolysis time (minutes)	HCl concentration (M)	Total flavonoid content (mg QE/g extract)	Total phenolic content (mg GAE/g extract)
1	15	30	0.3	97.07 ± 0.81	165.25 ± 2.47
2	25	30	0.3	64.45 ± 0.40	90.57 ± 1.91
3	15	30	0.9	66.76 ± 2.07	82.20 ± 2.92
4	25	30	0.9	83.79 ± 2.38	135.92 ± 7.35
5	15	15	0.6	83.48 ± 4.42	121.41 ± 5.09
6	25	15	0.6	79.66 ± 3.02	113.00 ± 11.33
7	15	45	0.6	88.20 ± 1.99	115.16 ± 3.12
8	25	45	0.6	81.23 ± 2.80	100.02 ± 2.18
9	20	15	0.3	85.65 ± 2.14	167.29 ± 9.96
10	20	15	0.9	75.32 ± 3.69	139.82 ± 5.13
11	20	45	0.3	75.83 ± 1.76	157.10 ± 6.45
12	20	45	0.9	84.02 ± 3.83	141.80 ± 5.04
13	20	30	0.6	106.28 ± 4.83	125.69 ± 6.89
14	20	30	0.6	107.65 ± 2.38	126.90 ± 3.75
15	20	30	0.6	106.62 ± 4.37	126.03 ± 2.35

As shown in the 3D plot of the TFC response, the graph forms a peak on the surface, indicating that the peak corresponds to the optimal X and Y values that yield the maximum response. This peak response signifies that the optimal conditions are at the maximum point. However, the 3D response plot for total phenolic content shows a wavy surface, with valleys forming at specific combinations of time and HCl concentration, indicating that the optimal point of X and Y yields the minimal response. Despite some studies suggesting that hydrolysis using HCl increases the phenolic compound content [12], the wavy surface response suggests otherwise in this case. Nevertheless, the statistical analysis confirms that the model used meets the requirements, with the quadratic model and ANOVA analysis satisfying the necessary criteria, thereby validating its use. These response results are mathematically predicted through the linear regression equation.

Next, we verify the RSM system's recommendations for the optimal conditions. During the analysis, the RSM system suggested seven optimal conditions. The one with the desirability value closest to the optimal value of 1 was selected. RSM suggests an extraction time of 17 minutes, a hydrolysis time of 15 minutes, and a HCl concentration of 0.5 M, predicting a total flavonoid content of 98.0 mg QE/g extract and a total phenolic content of 167.3 mg GAE/g extract.

The optimal conditions suggested by RSM were used as a reference for the extraction process, with variations in the pre-treatment process. These pre-treatment variations included (1) defatted and hydrolyzed (DH), (2) defatted but not hydrolyzed (DNH), (3) not defatted but hydrolyzed (NDH), and (4) neither defatted nor hydrolyzed (NDNH). According to the one-way ANOVA statistical test, the p-values for the four samples showed significant differences. However, post-hoc Tukey test analysis indicated that the significant difference ($P<0.05$) was between the defatting-hydrolysis group and the other three groups.

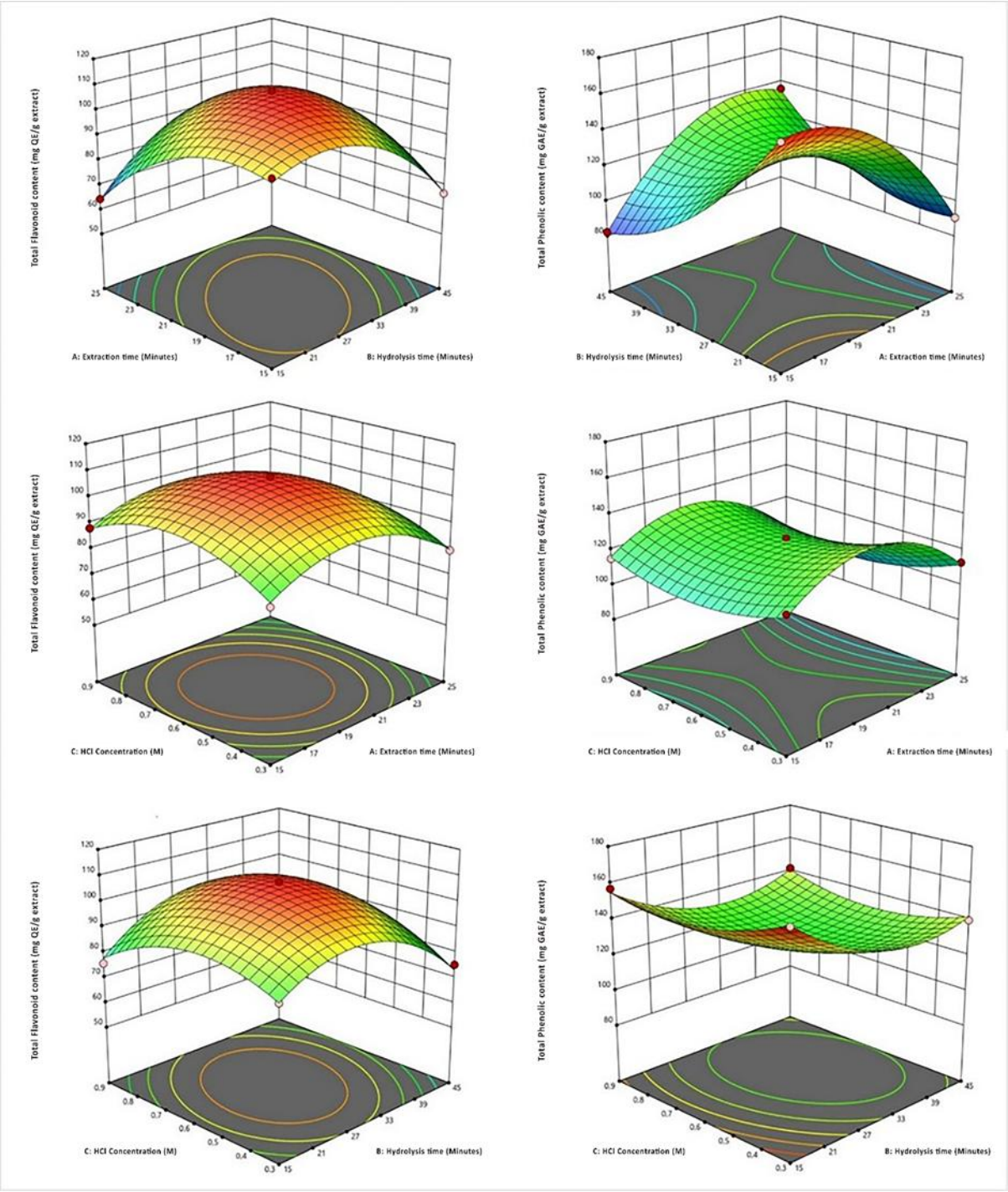

Figure 1. The 3D surface response diagram illustrates the effects of microwave extraction time, hydrolysis time, and HCl concentration on the total flavonoid content and total phenol content.

This study performed defatting using n-hexane, a lipophilic solvent with a log P value of 4 [13]. The solubility properties of kaempferol and apigenin compounds led to the selection of n-hexane as the defatting solvent. The log P values of kaempferol and apigenin are 2.2824 and 2.5768, respectively [14]. These log P values predict that n-hexane will not extract kaempferol and apigenin compounds. Additionally, defatting with n-hexane is expected to remove non-polar compounds, including alkaloids, terpenoids, chlorophyll, and fatty acids [13,15]. Defatting was performed using the maceration method, the simplest and most straightforward extraction technique, which can be conducted at room temperature. This method minimizes exposure to high temperatures, thereby preventing the degradation of heat-sensitive compounds.

Flavonoids exist in two forms: free form (aglycone flavonoids), which are soluble in low-polarity solvents, and glycosylated form (glycosides), which are soluble in high-polarity solvents, making them highly water-soluble. Glycosides in these leaves break down into their aglycone forms, thereby increasing the total flavonoid and phenolic contents. The cell wall surfaces in non-hydrolyzed extracts exhibited cracks, roughness, and unevenness, suggesting that the extraction process alters the plant cell wall structure, enabling the organic solvent to extract active compounds from the cells. After hydrolysis, the cell surface underwent a significant change, becoming a porous structure resembling a honeycomb. This suggests that hydrolysis leads to a substantial collapse of the cell wall, increasing the contact area between the solvent and the residue. Therefore, this increases the release of bound active compounds [16]. Catalysts in hydrolysis can be either acidic or basic. Acid hydrolysis results in higher phenol and flavonoid content compared to base hydrolysis [17]. HCl was selected as the catalyst because it is a strong acid that completely dissociates into H^+ ions in water. The more H^+ protons released, the easier it is to break the glycosidic bonds [10,18,19].

3.2. Validation of analytical method and determination of kaempferol and apigenin content in M. oleifera leaf extract simultaneously by HPLC.

The validation of the analytical method for determining kaempferol and apigenin in moringa leaf extract is essential, as a review of the literature indicates that no prior studies have addressed this topic. Kaempferol is more polar than apigenin; it has a stronger attraction to the polar mobile phase. As a result, kaempferol elutes earlier than apigenin. In the tests with the same mobile phase (50% A-50% B), kaempferol peaks appeared at 15.5 minutes and apigenin peaks appeared at 17.9 minutes. The two peaks were easily separated. Subsequently, system suitability tests and analytical method validation were conducted, including linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ).

In this study, the system suitability test was conducted through six injections, with observations on retention time, area under the curve, peak height, tailing factor, theoretical plate count, and resolution. The system suitability tests demonstrated excellent column efficiency, with theoretical plate counts ranging from 16,409 to 28,403, tailing factor values between 0.946 and 1.027, and resolution values exceeding 1.5. Furthermore, based on the data obtained, the %CV for all parameters met the requirements, indicating that the operational system was effective and produced results in line with the analytical objectives. Table 2 presents validation data for the simultaneous analysis method for kaempferol and apigenin.

Table 2. Validation data of the simultaneous analysis method of kaempferol and apigenin.

Parameters		**Kaempferol**	**Apigenin**
Linearity (R^2)		0.9999	0.9984
LOD		0.50 µg/mL	2.63 µg/mL
LOQ		1.65 µg/mL	8.76 µg/mL
Precision (% CV)	***Intraday***	0.68	0.32
	Interday	0.46	0.81
Accuracy (% Recovery)	Concentration 80%	100.75%±0.00	91.59%±0.00
	Concentration 100%	98.50%±0.00	95.04%±0.00
	Concentration 120%	99.23%±0.01	95.68%±0.01

3.3. Quantification of Moringa leaf extract samples.

The quantification of kaempferol and apigenin was determined using a spike method. The concentrations of kaempferol and apigenin in the four groups differed significantly ($p<0.05$), indicating that the defatting and hydrolysis processes affect the concentrations of

kaempferol and apigenin in leaf extract samples. The results of kaempferol and apigenin content measurement in *M. oleifera* leaf extract are shown in Figure 2A. Similar to the results of the total flavonoid content test, this variation arises from the hydrolysis of kaempferol and apigenin, initially present as glycosides, leading to an increase in the concentration of their aglycones.

The hydrolysis process in this study successfully cleaved the glycosidic bonds of kaempferol but did not effectively cleave those of apigenin. Increasing the HCl concentration did not guarantee a corresponding increase in apigenin aglycone levels, indicating the need for further optimization [20].

For kaempferol, hydrolysis appeared to reach completion at an HCl concentration of 2.5 mol/L, as indicated by the stabilization of kaempferol concentrations beyond this point. Temperature during hydrolysis also plays a significant role, influencing the concentration of active compounds.

3.4. HIV-1 RT inhibition activity test.

The HIV-1 RT inhibition activity was evaluated using two moringa leaf extract samples: DH and NDNH. Inhibition activity was assessed by IC_{50} values, as shown in Figure 2B. Kaempferol and apigenin were tested as active compounds due to their previously documented HIV-1 RT inhibition activity. Kaempferol exhibited the lowest IC_{50} value (41.75 µM), followed by apigenin (55.12 µM), consistent with previous findings [21,22].

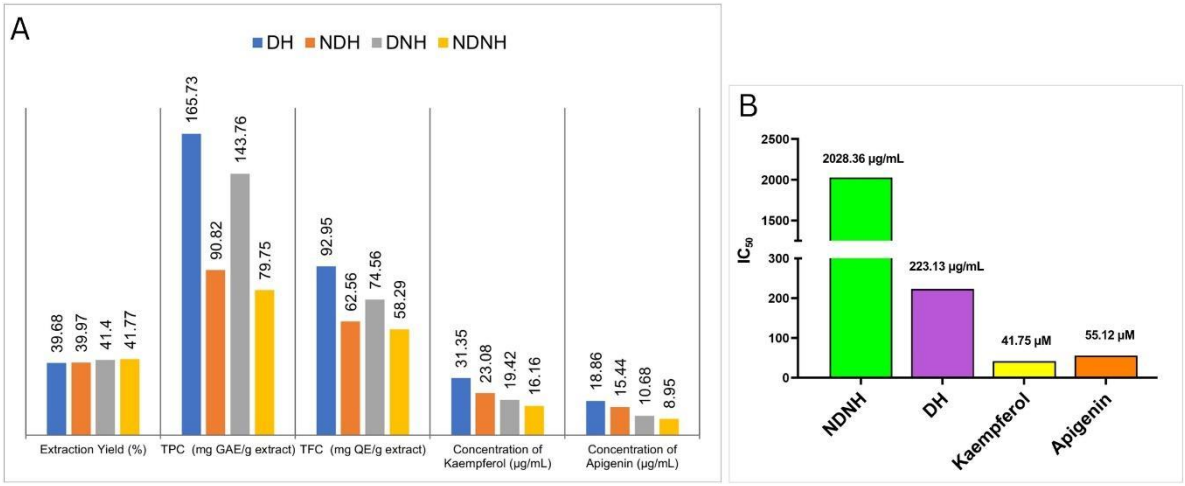

Figure 2. Results of **(A)** measurement in *M. oleifera* leaf extract; **(B)** IC_{50} values in the HIV-1 Reverse Transcriptase inhibition activity assay. DH = defatted and hydrolyzed; DNH = defatted but not hydrolyzed; NDH = not defatted but hydrolyzed; NDNH = not defatted and not hydrolyzed.

The results indicated that the DH sample inhibited reverse transcriptase activity, significantly higher (IC_{50} = 223.13 µg/mL) than the NDNH sample (IC_{50}>2000 µg/mL). According to the IC50 parameters, the NDNH sample was indicated as inactive, while the DH sample demonstrated moderate to minimal activity against the target [23]. This enhanced inhibition is likely due to the effects of defatting and hydrolysis on *the M. oleifera leaf extract, which contribute to higher* concentrations of kaempferol (0.78 µg/g) and apigenin (0.47 µg/g) compared to other treatments. This extract's activity remains moderate when compared to the inhibitory effects of pomegranate rind and guava leaf extracts on HIV-1 reverse transcriptase, which significantly inhibit HIV-1 RT activity, with IC_{50} values of 0.26 and 0.34 µg/mL, respectively [24].

However, while these compounds contribute to the observed inhibition, the overall activity of the moringa leaf extract is likely affected by the synergistic contribution of various phytochemicals present in the leaves, such as coumarin and vanillin, which are known to possess antiviral properties [25,26]. These synergistic effects suggest the potential to reduce the dosage or frequency of administration for either or both drugs, thereby minimizing potential toxicity and adverse effects. Further study is required to elucidate the dynamics of this synergy or antagonist and to ascertain whether the therapeutic efficacy of the entire extract is attributable to its multi-component synergy rather than to a singular active ingredient [27].

This study demonstrates the potential of bioactive compounds from natural products, such as Moringa oleifera, as promising sources of anti-HIV agents. Compounds such as kaempferol and apigenin may serve as lead compounds, with their pharmacological properties potentially improved through structural modifications. To facilitate sustainable development, an optimized extraction method is essential; the present findings indicate that microwave-assisted extraction (MAE), defatting, and hydrolysis can substantially enhance the yields of these active chemicals.

Although antiretroviral therapy has demonstrated efficacy by targeting essential viral enzymes—specifically reverse transcriptase, integrase, and protease—there remains a critical need for novel drugs that exhibit enhanced efficacy, reduced toxicity, and the capacity to overcome viral resistance. Rational drug design, structure–activity relationship (SAR) analysis, and advanced screening technologies will be crucial for expediting drug development. Consequently, additional *in vitro* and *in vivo* investigations are necessary to assess their therapeutic potential, followed by clinical trials in humans to guarantee safety and efficacy.

4. Conclusions

This study highlights the potential benefits of incorporating moringa leaf extract, which is abundant in kaempferol and apigenin, into the management of HIV/AIDS. Moringa leaves, known for their safety of consumption, may help inhibit viral replication due to their kaempferol and apigenin content. Additionally, extraction techniques such as defatting, microwave-assisted extraction (MAE), and hydrolysis can further enhance the level of kaempferol and apigenin in moringa leaf extract. Although the inhibition values of the moringa leaf extract are not particularly strong compared to pure kaempferol and apigenin, the extract's broad-spectrum biological activities may provide complementary benefits in therapeutic applications and offer more synergistic therapeutic options. Further study of the moringa leaves extract may be explored.

Author Contributions

Conceptualization, M.F., A.M., and F.; Methodology, M.F., A.M., F., M.R.I., N.B.N., and D.F.P.; Software, M.F.; Validation, M.F., A.M., and F.; Data curation, M.F., A.M. and F.; Formal analysis, M.F., A.M., and F.; Investigation, M.F., A.M., F., M.R.I., N.B.N., and D.F.P.; Resources, M.F.; Writing – original draft, M.F.; Writing – review & editing, M.F., A.M., F., and M.R.I.; Visualization, M.F., A.M., F., and M.R.I.; Supervision, A.M., and F.; Project administration, M.F., A.M., and F.; Funding acquisition, F.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Conflicts of Interest

The authors declare no conflict of interest.

Role of Funders

The funders had no role in the design of the study, in the collection, analysis, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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