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Enhancing the Cosmetic Potential of *Vitex trifolia* L. Extract: An Approach of Aqueous–Polyol Mixture in Ultrasound-Assisted Extraction for Wound-Healing and Antioxidant Properties

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ABSTRACT

This research aimed to optimize solvent concentration and extraction conditions for total phenolic content (TPC) and antioxidant activity (DPPH and ABTS) in VT leaves using ultrasound-assisted extraction (UAE). A simplex-lattice design incorporating an aqueous–polyol mixture and a central composite design (CCD) within response surface methodology (RSM) were utilized. The optimal solvent composition was determined to be 37.32% w/v aqueous, 26.87% w/v glycerin, and 35.81% w/v butylene glycol, with an ideal solvent-to-sample ratio of 33.21:1 mL/g and an extraction duration of 15 min. UAE with the aqueous–butylene glycol–glycerin (U-BG) mixture significantly enhanced TPC and antioxidant activity compared to conventional maceration methods ($p < 0.05$). Key bioactive compounds identified included Orientalol E, (+)-15-Beyeren-3-one, and petunidin-3-O-arabinoside. The U-BG extract exhibited higher cell viability and antioxidant activity compared with ethanol-based extracts and demonstrated strong wound-healing properties, achieving effective wound closure within 20 h. This study underscores the potential of aqueous–polyol extraction for enhancing bioactive compound recovery, offering sustainable solutions for the cosmetic and pharmaceutical industries.

1 | Introduction

A wound is a dermal injury that compromises skin integrity, initiating a sequence of related physiological processes that include hemostasis, inflammation, proliferation, and remodeling. These processes involve interactions among neutrophils, fibroblasts, endothelial cells, keratinocytes, and macrophages [1]. During the inflammatory phase, neutrophils and macrophages produce reactive oxygen species (ROS) to defend against infections [2]. In the proliferative phase, platelets, macrophages, and neutrophils promote the release of growth factors and cytokines, promoting keratinocyte and fibroblast migration and proliferation at the wound site [3]. Fibroblasts subsequently differentiate into myofibroblasts, leading to collagen production, tissue granulation, wound contraction, and remodeling [1]. Despite these well-orchestrated events, wound healing can be impaired by persistent inflammation, excessive ROS production, or microbial invasion. Recent studies highlight the importance of modulating oxidative stress, regulating growth factor signaling, and supporting extracellular matrix dynamics to promote

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effective tissue regeneration [4]. In this context, antioxidant-rich phytochemicals have gained considerable attention for their ability to reduce oxidative damage while enhancing cellular activities required for repair [5]. Moreover, natural product–based treatments offer practical advantages, including favorable safety profiles, accessibility, and lower cost, making them promising candidates for chronic wound management [6].

Vitex trifolia (VT), a tropical shrub in the Lamiaceae family, is widely distributed, particularly in northern Thailand, including Chiang Rai, where it is known as Kontee-sor. In traditional Thai medicine, VT is used to treat fever, headaches, dizziness, and cough [7]. The medicinal use of VT leaves is also prevalent in other countries such as New Caledonia, Rotuma, and the Solomon Islands, where they are applied heated, infused, or directly to the forehead for headache relief. In Tonga, VT is used to treat mouth infections and inflammation [8]. VT leaves and roots have been traditionally used to treat leprosy and skin rashes, particularly when combined with honey [7]. This plant contains bioactive compounds, including terpenoids, flavonoids, phenylpropanoids, phenolic acids, and steroids [9]. Research highlights VT's diverse biological properties, including anticancer, anti-inflammatory, hepatoprotective, antifungal, insecticidal, and analgesic effects [7, 10]. Consequently, plants of the Vitex genus have been traditionally used to manage ailments such as fever, diabetes, diarrhea, dysentery, flatulence, indigestion, and cholera [8]. These findings underscore VT's importance in traditional and modern medicinal applications.

Glycerin, propylene glycol, and butylene glycol (BG) are emerging as promising alternatives to conventional solvents in plant extraction, particularly within the framework of green chemistry. These polyols have gained attention for their potential to enhance the extraction of bioactive compounds while offering a more sustainable and environmentally friendly approach [11]. Polyols can be used as an alternative solvent, as they are commonly utilized in cosmetics and food industry, and are generally recognized as safe (GRAS) by the United States Food and Drug Administration (FDA). Similar to polar solvents, polyols assist in dissolving a variety of compounds [12]. In addition, the green extraction perspective has gained significant attention in plant extraction, utilizing solvents that are safe, nonflammable, sustainable, nonvolatile, cost-effective, easy to use, and recyclable. This approach aims to maximize the value of agricultural waste, by-products, and plant materials using environmentally friendly technology [13]. Furthermore, green extraction also involves the use of novel methods that include the development of new extraction processes and the replacement of organic solvents with alternative substances [14]. Prior research has demonstrated that ultrasound-assisted extraction (UAE) is successful in extracting bioactive compounds from plant leaves [15, 16], where extraction efficiency is greatly impacted by both acoustic power and treatment duration. Moreover, the short-term thermal exposure characteristics of UAE maintain the integrity of the extracted compounds, thereby enhancing the quality of the final product [17]. This provides various advantages from a green chemistry standpoint, including less solvent utilization, shortened extraction duration, and decreased energy consumption [17, 18].

Recent studies have explored the extraction of phenolic compounds from VT using various solvents and methods, including aqueous, hexane, ethyl acetate, dichloromethane, methanol, chloroform, and ethanol [19–21]. Additionally, previous research has utilized Soxhlet extraction for bioactive compound extraction from VT using ethanol for wound-healing applications [22]. However, research on UAE with an aqueous–polyol system (green solvents) for VT bioactive compound extraction and their application in wound healing has not yet been reported. This study focuses on optimizing the proportions of solvents in an aqueous–polyol system through a simplex-lattice mixture design. Additionally, it evaluates extraction parameters for total phenolic content (TPC) and antioxidant activity (DPPH and ABTS) using UAE from VT leaves through CCD in RSM. This study compares the conventional extraction techniques of UAE and maceration for the extraction of bioactive components using polyols (U-BG and M-BG) and ethanol (U-E and M-E) as solvents. Phenolic compounds in the extracts are identified using UHPLC-ESI-QTOF-MS/MS. Additionally, the extracts are assessed for cytotoxicity, cellular antioxidant activity, and wound-healing potential to evaluate their suitability for cosmetic applications. By integrating an eco-friendly extraction technique using green solvents, this research enhances the economic value of VT while promoting sustainability through improved bioactive compound extraction.

2 | Results and Discussion

2.1 | The Determination of the Suitable Concentration of the Solvent

Polyols, such as glycerin, BG, and propylene glycol, are commonly used in pharmaceutical and cosmetic formulations due to their humectant properties. In addition to hydration, glycerin enhances skin barrier regeneration by improving the integrity, stability, and mechanical properties of the stratum corneum. It also facilitates desmosomal degradation, aiding skin renewal [23, 24]. Higher solvent concentrations generally enhance the extraction of phenolic compounds, especially when the solvent's polarity corresponds to that of the target bioactive compounds. This emphasizes the significance of adjusting solvent concentration to enhance extraction efficiency. The type and concentration of the solvent are important in the extraction of phenolic compounds from plant extracts, mainly because of their polarity [25]. This study employed a simplex-lattice mixture design to evaluate glycerin, BG, and aqueous, optimizing solvent compatibility with plant bioactive compounds to enhance extraction efficiency. The TPC values ranged from 8.48 to 20.51 mg GAE/g sample (Table S1). The use of glycerol or glycols as extraction solvents enhances the yield of bioactive compounds while eliminating the need for an additional separation step from the solid matrix. This not only simplifies the extraction process but also reduces energy consumption during process scaling [26]. Regarding ternary mixture, the TPC was highest for volume ratios where the three solvents were evenly distributed. Comparable TPC content was obtained from the symmetrically distributed volume of three solvents, and the results of the aqueous mixture of B and G were higher compared to those of pure solvents. An increase in the yield of bioactive compounds extracted using aqueous–BG–glycerin solvent has been shown in previous research [27].

TPC data obtained from the experimental mixture design (Figure 1) were statistically evaluated using analysis of variance

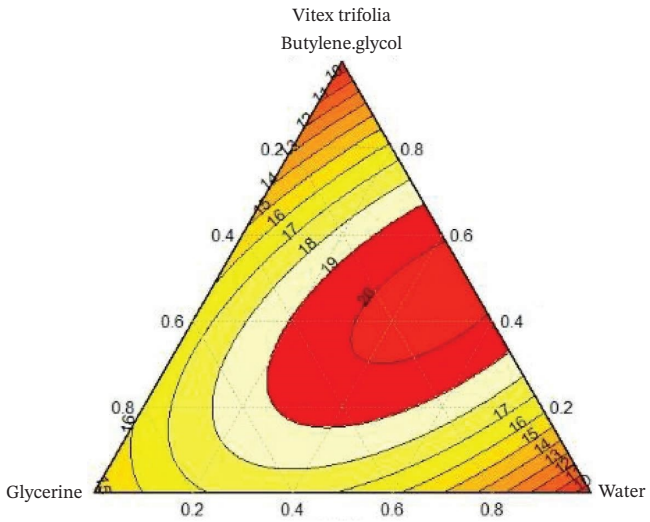

FIGURE 1 | Solvent concentration is optimized by simplex-lattice mixture design.

(ANOVA), followed by multiple regression modeling based on the polynomial equation presented in Equation [1]. This analytical approach aimed to assess how variations in the three solvent concentrations affected TPC levels.

$$\begin{aligned} \text{TPC} = {} & 9.17\text{A} + 14.79\text{B} + 9.23\text{C} \\ & + 15.66(\text{AB}) + 44.46(\text{AC}) + 19.65(\text{BC}). \end{aligned} \quad (1)$$

BG (A), glycerin (B), and aqueous (C) are the components included in the equation.

A p value below 0.05 and an F value of 347.42 indicate that the model is statistically significant. The coefficient of determination ($R^2 = 0.9957$) indicates a robust model fit, whereas the adjusted R^2 (0.9928) supports its reliability [28, 29]. The lack of fit was not statistically significant ($p \geq 0.05$), providing further evidence of the model's appropriateness (Table 1). The TPC was significantly influenced by all linear and interactive variables, including glycerin, BG, and aqueous components. Polyols function as effective cosolvents by modifying aqueous polarity, thereby enhancing polyphenol recovery [30]. Moreover, previous studies indicate that employing a combination of solvents improves the efficiency of bioactive compound extraction [31, 32]. The optimal solvent composition, identified using the simplex-lattice model, was 37.32% w/v aqueous solution, 35.81% w/v BG, and 26.87% w/v glycerin. This optimized solvent mixture was used in validation experiments, and the results, presented in Table 2, showed no statistically significant differences ($p \geq 0.05$).

2.2 | Determining the Solvent-to-Sample Ratio and the Extraction Duration

2.2.1 | The Influence of Extraction Duration

Extraction time is a key factor that influences the extent of solvent interaction with plant constituents, promoting the efficient release of bioactive compounds and enabling chemical interactions between the solvent and the plant matrix [27, 33]. Notably, the highest TPC was achieved with a 25-min extraction duration at 18.06 ± 0.96 mg GAE/g sample, whereas the lowest TPC was obtained with a 5-min duration at 14.18 ± 0.08 mg GAE/g sample ($p < 0.05$) (Figure 2). The present research shows that extraction duration directly correlates with the percentage of bioactive compounds extracted, suggesting that extended exposure times improve the transfer of these plant components into the solvent [33]. Therefore, a 25-min extraction period was selected for further experiments, as it yielded the highest TPC among all durations tested.

2.2.2 | The Influence of Solvent-to-Sample Ratio

According to Manzoor et al., the solvent-to-sample ratio provides a significant factor in determining the polyphenol yield [34]. There was a statistically significant increase ($p < 0.05$) in the TPC yield as the solvent-to-sample ratio increased to 30:1, where it reached its highest value (Figure 2). An increase in the solvent-to-sample ratio beyond 30:1 led to a reduction in TPC yield, aligning with trends reported in earlier research [35]. While a 30:1 solvent-to-sample ratio provides an optimal balance for

TABLE 1 | Response variance analysis in a simplex-lattice design.

	Df	Sum of squares	Mean square	F value	Pr ($>F$)
Model	6	3731.34	621.89	347.4246	$3.98e-10^{***}$
A	1	1715.68	1715.68	1178.953	$3.941e-07^{***}$
B	1	1129.96	1129.96	776.4654	$1.114e-06^{***}$
C	1	656.23	656.23	450.9368	$4.293e-06^{***}$
AB	1	15.52	15.52	10.6626	0.022313^{*}
AC	1	188.34	188.34	129.4169	$9.184e-05^{***}$
BC	1	25.6	25.6	17.5928	0.008536^{**}
Residuals	9	16.11	1.79		
Lack of fit	4	8.84	2.21	1.5178	0.324989
Pure Error	5	7.28	1.46		
Total	15	3747.45			
R-squared	0.9957				
Adj. R-squared	0.9928				

Note: Asterisks denote levels of statistical significance, where $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$.

TABLE 2 | The results of validating the expected and observed values on TPC from VT.

Conditions	Butylene glycol (%w/v)	Glycerin (%w/v)	Aqueous (%w/v)	TPC (mg GAE/g sample)
Expected	35.812	26.865	37.323	20.121
Observed	35.81	26.87	37.32	20.59 ± 0.44^{ns}

Note: Data are represented as mean ± SD ($n = 3$).
[ns] Similar columns have no significant differences ($p \geq 0.05$).

phenolic extraction, increasing the ratio to 45:1 results in greater dilution of phenolic compounds in the solvent. This dilution lowers the concentration of extracted phenolics per unit volume, potentially reducing extraction efficiency and affecting measurement accuracy. Moreover, at a low solvent-to-sample ratio, variations in extraction efficiencies are generally insignificant; therefore, this parameter is typically optimized to a specific value [36]. The findings of this study are consistent with those of Goula et al. and Manzoor et al., who reported that an optimal solvent-to-sample ratio enhances the extraction yield of phenolics from pomegranate waste and marigold flower petals. However, further increasing the ratio did not result in significant improvements [34, 37].

2.3 | Optimization of Extraction Utilizing Response Surface Methodology

The optimization procedure for the extraction of TPC and assessing antioxidant activity (DPPH and ABTS) is carried out using RSM. The outcomes of different assays (TPC, DPPH, and ABTS) for VT extract are shown in Table S3.

2.3.1 | The Model of VT Leaf Extraction Conditions

The data in Table S3 were analyzed by multiple regression fitting and quadratic polynomial equations [2–4] that demonstrate the relationship between the two variables (extraction time and solvent-to-sample ratio) and responses (TPC, DPPH, and ABTS).

$$\mathrm{TPC} = 15.513 + 1.207\mathrm{A} + 1.513\mathrm{B} - 0.001\mathrm{AB} - 0.211A^2 - 2.173B^2, \quad (2)$$

$$\mathrm{DPPH} = 20.226 + 2.064\mathrm{A} + 5.313\mathrm{B} + 1.182\mathrm{AB} - 0.494A^2 - 2.686B^2, \quad (3)$$

$$\mathrm{ABTS} = 39.391 + 3.231\mathrm{A} + 10.440\mathrm{B} + 1.119\mathrm{AB} - 0.996A^2 - 4.120B^2. \quad (4)$$

Extraction duration (A) and solvent-to-sample ratio (B) are defined in the equation.

Data analysis was conducted using R software (Version 4.4.1) with a quadratic polynomial regression model, incorporating TPC, DPPH, and ABTS as response variables. The results indicate that all three models were statistically significant ($p < 0.05$), demonstrated by F values of 16.84, 29.31, and 18.99, respectively (Table S4). Over 90% of the data was explained by the TPC, DPPH, and ABTS models, as the coefficients of determination (R^2) were more than 0.91 [38]. Model reliability was demonstrated by adjusted R square (adj R^2) values greater than 0.90 in DPPH, which indicates that there are minimal significant variations between adjusted and predicted values. The regression model for TPC, DPPH, and ABTS demonstrates a strong correlation between R^2 and

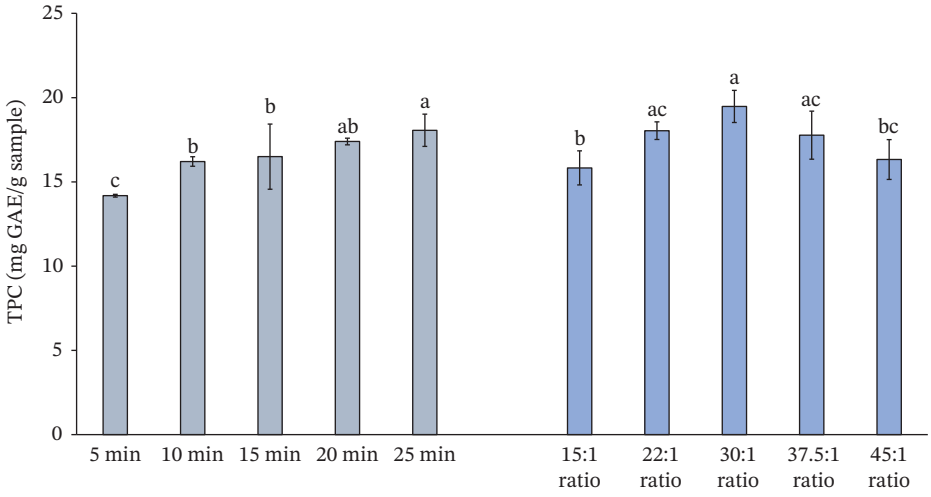

FIGURE 2 | The effect of extraction duration and solvent-to-sample ratio on total phenolic content utilizing UAE of VT leaves. Statistically significant differences among values are indicated by distinct superscript letters ($p < 0.05$). Data are represented as mean ± SD ($n = 3$).

adjusted R^2, as the difference between these values is minimal [39]. The models were appropriate, while the lack of fit terms was not statistically significant ($p \geq 0.05$).

2.4 | Impact of Independent Variables on TPC and VT Antioxidant Efficacy

This study investigated the effects of extraction duration and solvent-to-sample ratio on TPC and antioxidant activities by analyzing the results from 14 experiments. The findings, presented in Table 3 and Figure 3, illustrate the interaction effects on TPC, DPPH, and ABTS [a–c]. Enhancing both the extraction duration and the solvent-to-sample ratio produced a significant positive impact on all measured responses ($p < 0.05$), indicating that these factors boost overall extraction efficiency. In contrast, their interaction did not significantly influence TPC or antioxidant activity (DPPH, ABTS) ($p \geq 0.05$). The quadratic term for the solvent-to-sample ratio showed a significant negative coefficient ($p < 0.05$), revealing a curved response surface: yields increased with higher solvent ratios up to an optimal point, after which further increases failed to improve extraction (Figure 3). This leveling off likely arises because, as the solvent becomes increasingly saturated with phenolic compounds, its capacity to dissolve additional bioactives reaches a plateau or even diminishes [35]. Once bioactive compound concentrations in plant material were reduced, additional solvent did not improve yield [34]. Additionally, prolonged extraction durations may cause polyphenol degradation, highlighting the importance of optimizing extraction duration [40]. Studies on UAE of coffee pulp and *Canthium horridum* (CH) leaves using aqueous–BG mixtures revealed that TPC, DPPH, and ABTS values remained stable over extended extraction periods. As the solvent-to-sample ratio increased, these values initially rose until reaching saturation, beyond which further increases in solvent volume had no additional effect [27, 41]. Similarly, Pandey et al. reported no significant effect of extraction duration on TPC, ABTS, and DPPH. In contrast, they observed a positive linear effect on DPPH values and a negative linear effect of solvent-to-sample ratio on ABTS values. Overall, the solvent-to-sample ratio had a more substantial impact on extraction efficiency than extraction duration [42].

2.5 | Expected Value Validation

A solvent-to-sample ratio of 33.21:1 mL/g and an extraction duration of 15 min were shown to be the optimal parameters by means of the response model. Although the highest experimental TPC was observed at the prolonged extraction time, the RSM optimization selected 15 min as the ideal extraction time. This shorter duration provided a better balance between phenolic yield, extraction efficiency, and process sustainability. Extending the extraction beyond 15 min resulted in only minimal increases in TPC while potentially promoting the thermal or oxidative degradation of phenolic compounds, along with unnecessary energy use [36]. Thus, the model identified 15 min as the most efficient and stable operating point for UAE. The validation experiment was conducted to evaluate the precision and accuracy that the response model has under these optimal conditions. The experimental results were compared to the predicted values (Table 4). With p values greater than 0.05, there were no discernible differences between the predicted and actual results. The results indicate that the response model for that extraction technique is reliable.

TABLE 3 | Responses in the central composite design analyzed using regression.

Source	TPC				DPPH				ABTS			
	Coefficient estimated	Standard error	*t* value	*p* value	Coefficient estimated	Standard error	*t* value	*p* value	Coefficient estimated	Standard error	*t* value	*p* value
Intercept	15.51289617	0.35823186	43.3041	$8.919e-11^{***}$	20.22625	0.60275	33.5569	$6.790e-10^{***}$	39.39051	1.38277	28.4866	$2.493e-09^{***}$
A	1.20711556	0.31023789	3.8909	0.0046031^{**}	2.06432	0.52199	3.9547	0.004208^{**}	3.23109	1.19752	2.6982	0.02715^{*}
B	1.51260323	0.31023789	4.8756	0.0012312^{**}	5.31295	0.52199	10.1782	$7.438e-06^{***}$	10.43990	1.19752	8.7179	$2.340e-05^{***}$
AB	−0.00096061	0.43874263	−0.0022	0.9983067	1.18200	0.73821	1.6012	0.148006	1.11913	1.69355	0.6608	0.52730
A^2	−0.21147870	0.32290583	−0.6549	0.5308926	−0.49401	0.54331	−0.9093	0.389781	−0.99551	1.24642	−0.7987	0.44752
B^2	−2.17265134	0.32290583	−6.7284	0.0001483^{***}	−2.68640	0.54331	−4.9445	0.001129^{**}	−4.12002	1.24642	−3.3055	0.01077^{*}

Note: Asterisks denote levels of statistical significance, where $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$.

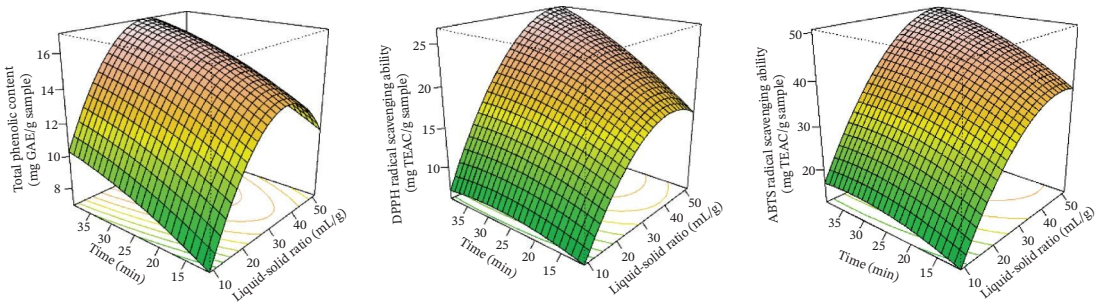

FIGURE 3 | The effects of solvent-to-sample ratio and extraction duration on TPC (a), DPPH (b), and ABTS (c) shown in response surface plots.

2.6 | Comparison of UAE With Conventional Method Utilizing Polyol-Based Solvents or Ethanol

A summary of the results from four different extraction methods—U-BG, U-E, M-BG, and M-E—applied to VT leaves under conditions optimized by the response surface model is presented in Table 5. Among these extracts, U-BG showed significantly higher efficiency in both TPC and ABTS radical scavenging activity ($p < 0.05$) compared to the other methods. M-BG ranked second in overall performance for most parameters, except for DPPH, where U-E recorded the second highest value. In contrast, M-E consistently yielded the lowest results across all metrics. It is noteworthy that the differences in DPPH activity among the extraction methods were not statistically significant ($p \geq 0.05$). Despite clear differences in TPC and ABTS activity, the DPPH scavenging values did not vary significantly among the four extraction methods. This consistency may reflect the chemical specificity of the DPPH assay, which predominantly responds to hydrogen-donating antioxidants and is less influenced by structural diversity within phenolic subclasses [43, 44]. It is also possible that the key compounds capable of reducing DPPH radicals were efficiently extracted across all methods, resulting in comparable activity. Additionally, nonphenolic antioxidants—such as certain terpenoids or reducing sugars—may contribute to radical quenching in a manner that is relatively insensitive to extraction conditions [45]. A previous study demonstrated that prolonged maceration times may lead to polyphenol degradation, reducing their bioactivity and effectiveness [36]. Myo and Khat-udomkiri concluded that plant extracts obtained using an aqueous–glycerin–BG solvent exhibited higher antioxidant activity and greater bioactive compound yields compared to those extracted with ethanol [27]. Furthermore, using the same extraction solvent, UAE consistently surpassed maceration across all parameters and required significantly less time. The effectiveness of UAE and maceration was evaluated in a prior study of bioactive compound extraction from *Echinacea purpurea* aerial parts using an aqueous–glycerin solvent. The results showed that both methods were equally effective, with the exception that UAE utilized shorter extraction time [46]. Our findings are supported by a previous study that demonstrated polyols produce more antioxidant-rich bioactive compounds than ethanol, regardless of their production method, maceration, or UAE. Moreover, UAE is recognized as an eco-friendly technique that delivers high extraction yields within a relatively short processing time [27, 47, 48].

2.7 | Determination of Bioactive Compounds by LC-QTOF-MS/MS

The identification of bioactive components in the VT leaf extract through LC-QTOF-MS/MS analysis is essential for understanding its pharmacological potential, particularly for skin-related conditions. UHPLC-ESI-QTOF-MS/MS in both positive and negative ionization modes was used to thoroughly identify the phenolic compounds in VT leaf extracts (Figure S1). The compounds were confirmed using MS/MS fragmentation patterns and Molecular Feature Generator (MFG) scores, which ensure accurate identification. Reliable identification was based on MFG scores above 80 and mass errors under 5 ppm. The LC-QTOF-MS/MS analysis revealed a diverse and complex range of bioactive compounds across various major classes, highlighting the rich chemical composition and potential health benefits of VT leaves (Figure S2). Among the identified compounds, arjunglucoside I and benzoic acid were previously reported, while all other compounds, including various phenolics, flavonoids, alkaloids, and terpenoids, were newly identified in VT leaf extracts (Table S5). Phenolic compounds, such as 2,4-dinitroresorcinol and glucosyringic acid, were prominent in U-BG and are well known for their antioxidant and anti-inflammatory properties, making them critical for reducing oxidative stress and inflammation in skin disorders [49, 50]. Newly identified flavonoids, including petunidin-3-O-arabinoside and 7-hydroxy-3-methoxy-1-primeverosyloxyxanthone, are recognized for their ability to modulate inflammatory pathways and protect against ROS [51, 52]. Additionally, saponins, such as arjunglucoside I, demonstrate immune-modulating and anti-inflammatory properties, further emphasizing their role in promoting skin healing and regeneration [53]. The study also identified alkaloids like N-phenylpiperazine-1-carboxamide, which exhibit antimicrobial and anti-inflammatory activities, suggesting their potential in combating skin infections and inflammatory disorders [54]. Furthermore, this study identified the presence of terpenoids, including orientalol E and (+)-15-beyeren-3-one, as terpenoids which possess analgesic, anti-inflammatory, and tissue-regenerating properties, which are essential for wound healing and inflammation management [55, 56]. This study is particularly novel as it represents the first comprehensive report on the bioactive profiling of VT leaf extract using a polyol–aqueous system under UAE, along with its in vitro and cellular activity assays. These findings not only highlight the pharmacological relevance of the compounds for skin health but also demonstrate the potential of sustainable extraction methods for valorizing

TABLE 4 | Expected value validation results with a 95% confidence interval.

Conditions	Extraction duration (min)	Solvent-to-sample ratio (mL/g)	TPC (mg GAE/g sample)		DPPH (mg TEAC/g sample)		ABTS (mg TEAC/g sample)	
			Mean ± SD	Effect sizes (95% CI)	Mean ± SD	Effect sizes (95% CI)	Mean ± SD	Effect sizes (95% CI)
Expected	15	33.21:1	14.32	1.32 (−0.38,2.91)	18.44	0.95 (−0.53, 2.31)	37.86	−0.78 (−2.06,0.61)
Observed	15	33.21:1	14.76 ± 0.33^{ns}		20.84 ± 2.53^{ns}		37.06 ± 1.03^{ns}	

Note: Data are represented as mean ± SD ($n = 3$).
[ns]Similar columns have no significant differences ($p \geq 0.05$).

underutilized plant parts, aligning with green chemistry principles and offering eco-friendly solutions for dermatological and pharmaceutical applications.

2.8 | Cell Culture

2.8.1 | Cytotoxicity Assay on the Keratinocyte

Cytotoxicity was determined using various concentrations of ascorbic acid (AA), allantoin (AT), and VT extracts to determine their maximum nontoxic concentration. For HaCaT keratinocytes, AA concentrations at 0.01, 0.05, and 0.1 mg/mL were regarded as noncytotoxic, with cell viability remaining above 80% (Figure 4). However, concentrations over 0.5 mg/mL suppressed cell survival. Higher AA levels demonstrated the capacity to induce cytotoxicity through the induction of metabolic stress, potentially resulting in apoptosis [57]. AT concentrations between 10 and 1000 μg/mL were also classified as noncytotoxic, with cell viability values of 94.57 ± 4.54%, 91.61 ± 0.85%, 98.79 ± 0.81%, 100.06 ± 3.71%, and 92.81 ± 3.12%, respectively. Similarly, U-BG extract at concentrations of 5 and 10 mg/mL was regarded as noncytotoxic, as cell viability remained above 80% [58]. Conversely, all tested U-E extract concentrations, except for 5 mg/mL, exhibited statistically significant cytotoxic effects ($p < 0.05$). A previous study demonstrated the dose-dependent cytotoxicity of VT extracts in MCF-7 cells, with petroleum ether at a concentration of 125 μg/mL and methanol at 500 μg/mL or higher, as determined by the MTT assay [59]. The cytotoxicity of VT leaf extracts varied by solvent, with methanol, ethanol, and aqueous extracts exhibiting higher IC_{50} values than hexane and dichloromethane. The Soxhlet aqueous extract exhibited the highest IC_{50} (684.5 ± 99.0 μg/mL), indicating solvent-dependent effects on cell viability [19]. Additionally, this study identified the presence of alkaloids, including aspidospermine, aspidocarpine, and uleine, in M-E. These compounds have been reported to exhibit toxicity against the HepG2 human hepatoma cell line [60, 61]. Our results from the cell viability assessment in HaCaT keratinocyte culture were higher in the BG extract than in the ethanolic extract. Similarly, in a study conducted by Myo and Khat-udomkiri in 2024, it was found that polyols had a lower cytotoxic effect compared to ethanol in cell culture [27]. Furthermore, the results are consistent with a recent study evaluating the UAE where extraction with BG solvent of *Camellia sinensis* flowers showed greater cell viability than the ethanol extract when both were exposed to similar conditions [48].

In this study, U-BG and MAR extracts (M-BG and M-E) exhibited a dose-dependent effect on HaCaT keratinocytes. Concentrations between 5 and 20 mg/mL were not toxic to cells, while U-E was noncytotoxic at 5 mg/mL. Thus, all noncytotoxic extracts were selected for cellular antioxidant and wound-healing (scratch) assays.

2.8.2 | Cytoprotective Efficacy of VT Extracts Against H_2O_2-Induced Oxidative Stress

Dose–response studies were conducted to determine the cytoprotective effect of extracts obtained from VT leaves (U-BG, U-E, M-BG, and M-E), and AA against H_2O_2-induced oxidative stress. Oyerinde et al. reported that the cellular antioxidant activity of all extracts was tested in an experiment where oxidative stress induced by H_2O_2 led to cell death due to DNA damage [62]. The cytoprotective effects of the extracts against 300 mM H_2O_2-

TABLE 5 | Comparison of UAE and maceration using polyol-based solvents and ethanol.

Conditions	Extraction duration	Solvent-to-sample ratio (mL/g)	TPC (mg GAE/g sample)	DPPH (mg TEAC/g sample)	ABTS (mg TEAC/g sample)
U-BG	15 min	33.21:1	14.76 ± 0.33^{a}	20.84 ± 2.53^{ns}	37.06 ± 1.03^{a}
U-E	15 min	33.21:1	13.16 ± 0.40^{bc}	20.53 ± 2.34^{ns}	29.60 ± 1.19^{b}
M-BG	24 h	33.21:1	13.56 ± 0.26^{b}	18.19 ± 1.15^{ns}	29.98 ± 1.82^{b}
M-E	24 h	33.21:1	12.28 ± 0.96^{c}	16.13 ± 2.84^{ns}	28.31 ± 1.08^{b}

Note: Data are represented as mean ± SD ($n = 3$). Statistically significant differences among values are indicated by distinct superscript letters within the same column ($p < 0.05$).
[ns]Similar columns have no significant differences ($p \geq 0.05$).

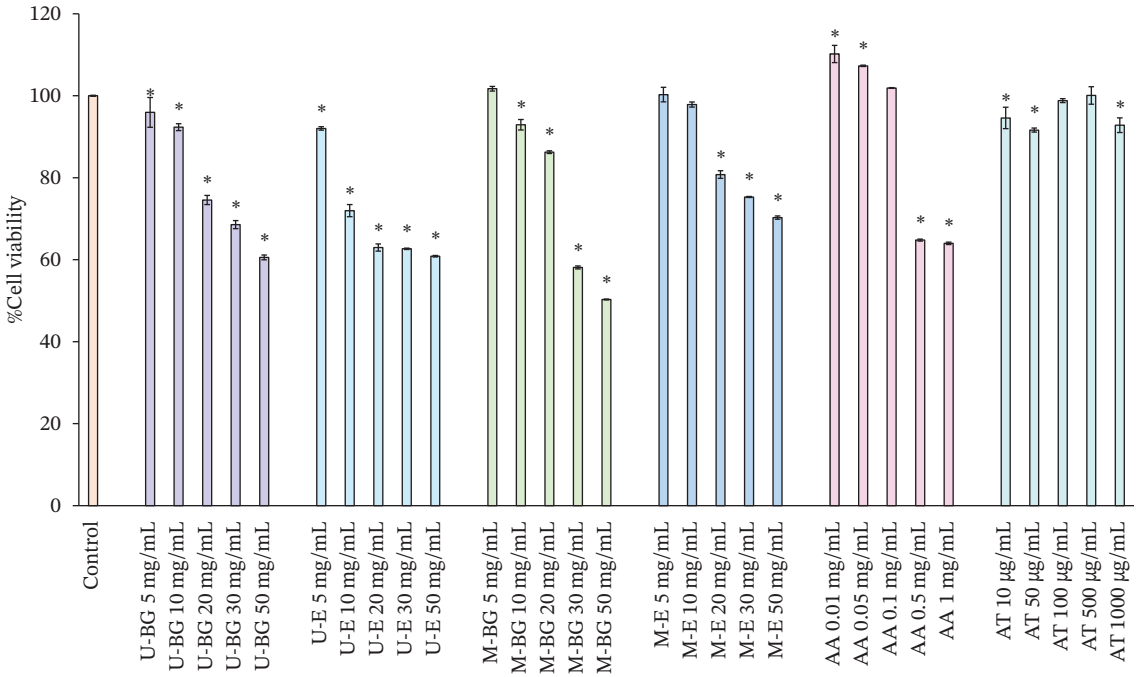

FIGURE 4 | Evaluation of cytotoxicity for U-BG, U-E, M-BG, M-E, ascorbic acid (AA), and allantoin (AT). Asterisks (*) indicate statistically significant differences compared to the control ($p < 0.05$). Data are presented as mean ± SEM ($n = 3$).

induced oxidative stress in HaCaT cells are shown in Figure 5. H_2O_2 treatment significantly reduced cell viability to 57.13 ± 0.81% compared to the control ($p < 0.05$) (Figure 5). Nonetheless, treatment with concentrations at 0.01 mg/mL and 0.05 mg/mL of AA markedly enhanced cell viability to 79.44 ± 0.31% and 75.09 ± 0.64%, respectively. Notably, both U-BG and U-E extracts significantly increased cell viability compared to H_2O_2 treatment alone ($p < 0.05$), with U-BG showing antioxidant activity comparable to U-E at the same concentration. Additionally, M-BG extract exhibited higher cell viability than M-E extract. The study identified phenolic compounds, such as glucosyringic acid, in UAE extracts (U-BG and U-E), which demonstrated the highest antioxidant activity with an IC_{50} of 18.11 μM [63]. Similarly, previous research on H_2O_2-induced oxidative stress in HDFs found that increasing embelin concentrations decreased cell viability, suggesting that high concentrations of M-BG extract and H_2O_2 may reduce cell viability [64]. In addition, prolonged maceration may promote the degradation of bioactive constituents, particularly phenolic compounds. This degradation can result in the accumulation of oxidized or structurally altered metabolites that exhibit pro-oxidant rather than antioxidant behavior. At elevated concentrations, such changes may contribute to cytotoxic effects, as observed in the M-BG and M-E extracts [36, 65]. At low to moderate concentrations, AA functions as an antioxidant by neutralizing ROS. At higher concentrations, it may act as a pro-oxidant, resulting in decreased cell viability [66]. A study on NIH/3T3 fibroblasts reported that an aqueous–glycerin–BG extract of *Canthium horridum* Blume leaves provided superior cytoprotection and viability compared to an ethanol extract. These findings underscore the potential of BG extracts in enhancing cellular defense against oxidative stress [27].

2.8.3 | Wound Closure on the Keratinocyte

The wound-healing potential was evaluated by measuring the percentage of wound closure, which was determined by comparing the gap size in each well at 0 and 20 h postscratch. Images of gaps were captured, and wound closure at 20 h was calculated by subtracting the gap size at 20 h from that at 0 h (Table S6). The

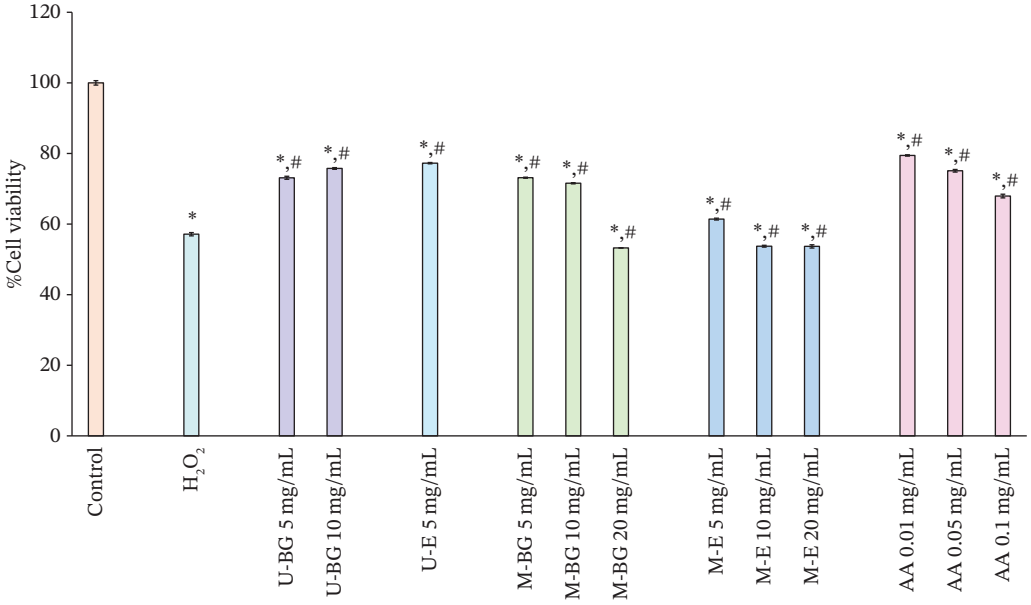

FIGURE 5 | Evaluation of the antioxidant properties of U-BG, U-E, M-BG, M-E, and ascorbic acid (AA). Asterisks (*) indicate statistically significant differences compared to the control ($p < 0.05$). The symbol "#" indicates a statistically significant difference relative to the H_2O_2 group ($p < 0.05$). Data are presented as mean ± SEM ($n = 3$).

results indicate that U-BG at a concentration of 10 mg/mL significantly enhanced wound closure in HaCaT cells ($p < 0.05$) compared to the untreated control and AT (100 μg/mL) (Figure 6). Similarly, M-BG at 5 mg/mL exhibited a significantly increased rate of wound-healing activity ($p < 0.05$) in comparison to the untreated control and AT. Additionally, U-E and M-E extracts at 5 mg/mL also significantly promoted wound-healing properties ($p < 0.05$) relative to the untreated control. In comparison to the untreated control, wound closure increased significantly ($p < 0.05$) after 20 h with AT (the positive control at 500 μg/mL). These findings suggest that U-BG and M-BG extracts possess strong wound-healing properties, facilitating effective wound closure within 20 h of treatment. The M-BG and M-E extracts showed a concentration-dependent reduction in the percentage of wound closure. This effect may be attributed to the nonselective nature of maceration extraction, which can lead to the co-extraction of undesirable constituents and potential contaminants. At higher concentrations, some of these co-extracted compounds may exert cytotoxic or antiproliferative effects, thereby impairing the wound-healing response [36]. Further analysis using LC-Q-TOF-MS revealed that the U-BG extract contained phenolic compounds, including orientalol E

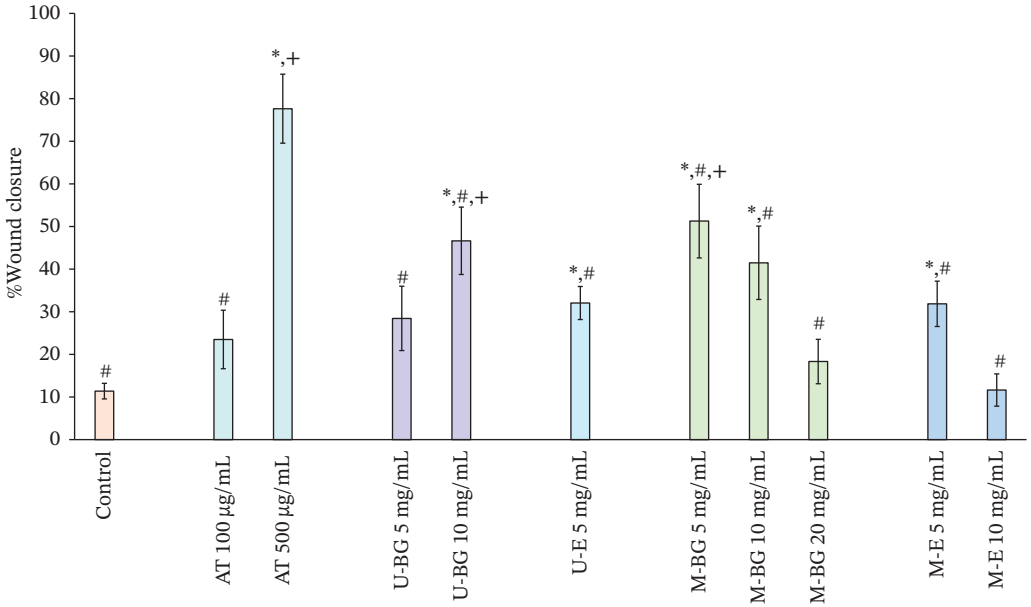

FIGURE 6 | Evaluation of wound healing for U-BG, U-E, M-BG, M-E, and allantoin (AT). Asterisks (*) indicate statistically significant differences compared to the control ($p < 0.05$). The symbol "#" indicates a statistically significant difference as compared to allantoin at 500 μg/mL ($p < 0.05$). The symbol "+" indicates a statistically significant difference compared to allantoin at 100 μg/mL. Data are presented as mean ± SEM ($n = 3$).

and (+)-15-beyeren-3-one, at higher levels than other extracts. Since these terpenoids have been reported to promote wound healing and modulate inflammation [55, 56], their presence may explain the enhanced wound closure observed in this study. In previous research, the ethanol extract of VT leaves demonstrated optimal wound-healing efficacy in Swiss Wistar strain rats, comparable to the control group [22]. Beyond the contribution of antioxidant terpenoids, the enhanced wound-healing activity of the U-BG extract may also involve the regulation of cellular mechanisms fundamental to re-epithelialization. Phenolic compounds that mitigate oxidative stress can stimulate keratinocyte migration and proliferation—two essential processes during the early phases of wound repair. These effects may activate MAPK and PI3K/Akt signaling pathways, promoting cytoskeletal reorganization and enhanced cell motility [67]. Additionally, several phytochemicals identified in the extract, such as petunidin-3-O-arabinoside, have been reported to upregulate growth factors including VEGF, TGF-β, and EGF in related plant systems. Such pathways support extracellular matrix remodeling and accelerate wound closure. Collectively, these mechanisms help explain the enhanced scratch-wound recovery observed in keratinocytes treated with the U-BG extract. Dysregulated inflammation or excessive cellular hyperplasia can hinder effective tissue repair. Therefore, future studies should investigate the incorporation of this extract into advanced delivery systems, such as microneedle platforms, to further optimize wound-healing outcomes [68]. Although the observed wound-healing and antioxidant effects are consistent with mechanisms reported for phenolic and terpenoid constituents, the mechanistic interpretations presented here remain inferential. Direct molecular validation—such as analysis of signaling pathway activation, gene expression, or protein-level responses—will be necessary in future studies to confirm the precise biological pathways underlying these effects.

3 | Conclusion

The studies presented utilized simplex-lattice and CCD within RSM to optimize UAE parameters for VT leaves. The optimal solvent composition was 35.81% w/v BG, 26.87% w/v glycerin, and 37.32% w/v aqueous, with ideal extraction conditions of a 33.21:1 mL/g solvent-to-sample ratio and a 15-min extraction duration. The extraction efficiency of VT was primarily influenced by the type of solvent, its concentration, extraction duration, and the solvent-to-sample ratio. Validation processes confirmed the reliability of the response model. An analysis of comparative performance demonstrated that U-BG outperformed U-E in all responses. Eighteen bioactive compounds were identified in the UHPLC-ESI-QTOF-MS/MS analysis, representing the initial LC-MS investigation of VT leaf extracts. In the cell cytotoxicity and antioxidant assay, U-BG exhibited enhanced cell viability and cellular antioxidant activity in response to hydrogen peroxide than U-E. Furthermore, by enhancing wound closure following 20 h of treatment, U-BG showed significant wound-healing properties, implying potential applications in cosmetic formulations (anti-inflammatory products) and pharmaceutical applications (wound-healing treatment). The authors acknowledge certain limitations in the investigation of bioactive compounds in VT leaves, particularly regarding their wound-healing properties, such as antimicrobial and anti-inflammatory effects, and the underlying mechanisms. It is recommended that additional research be conducted to investigate the mechanisms underlying these bioactive compounds using complex analytical and molecular biology methodologies. Furthermore, clinical trials are recommended to verify the safety and efficacy of the compounds while also optimizing the extraction process for industrial utilization. This study provides support for the wider application of an aqueous–polyol technique for the extraction of bioactive compounds from a variety of plants, including VT leaves. It also highlights the potential of these compounds for use in pharmaceutical and cosmetic formulations, notably in the treatment of wound healing and inflammation.

3.1 | Experimental Section

3.1.1 | Chemical Materials and Plant Materials

Botanists authenticated VT leaves collected in November 2019, and the Herbarium of Mae Fah Luang University received a voucher specimen (MFU-00616). All the chemicals were of analytical grade, except Chanjao Longevity Co. supplied the cosmetic grade of polyols. According to Supjaroenporn et al., the leaves were subjected to a 24-h drying process at 50°C. After that, they were ground into a fine powder and kept at room temperature for the next experiments [69].

3.1.2 | Determination of the Appropriate Concentration of Solvent

Table S1 presents the results from a simplex-lattice mixture design experiment aimed at identifying the optimal solvent concentration within an aqueous glycerin–BG system. The design included a total of 15 experimental runs. The efficacy of extraction was measured using the TPC, and the solvent mixes that significantly affected the extraction were identified using ANOVA [27].

3.1.3 | UAE

To extract VT leaves, the experiment was run continuously at room temperature according to the specified method [48]. An ultrasonic processor (VCX 130, Vibra-Cell, Sonics, USA) equipped with a 6-mm probe and operating at a constant frequency of 20 kHz was used. During sonication, the process was performed at a 50% duty cycle. After completion of the extraction, the resulting mixture was centrifuged at 2490 × g for 15 min to obtain the supernatant for further analysis.

3.1.4 | Determination of Extraction Duration and Solvent-to-Sample Ratio

Key extraction parameters, including the solvent-to-sample ratio (ranging from 15:1 to 45:1 mL/g) and extraction time (ranging from 5 to 25 min), were identified through the experimental setup. In each experiment, one independent variable was systematically varied while keeping all other parameters constant.

TPC was then measured to determine which parameters significantly influenced extraction efficiency. Specifically, the extraction time was varied while keeping the ratio constant at 30: 1 mL/g, and conversely, the solvent-to-sample ratio was varied while maintaining a fixed extraction time of 25 min.

3.1.5 | Experimental Design for Optimizing Extraction Methodologies

The extraction parameters for bioactive compounds from VT leaves were optimized using RSM combined with a CCD. Table S2 outlines the five coded levels for factors A (extraction duration, min) and B (solvent-to-sample ratio, mL/g). To develop the predictive model, TPC alongside DPPH and ABTS radical scavenging activities were measured across 14 experimental runs, as detailed in Table S3.

3.1.6 | Determination of the TPC, DPPH, and ABTS

The TPC and antioxidant activity (DPPH and ABTS assays) were assessed using the specified methodology [70].

3.1.7 | Validation of the Predicted Value

The reliability and precision of the established response model were evaluated by validating it under the optimized extraction conditions identified by CCD.

3.1.8 | A Study Comparing UAE With Maceration in Ethanol or a Selected Solvent

The extraction efficiency of UAE was compared with conventional maceration extraction using ethanol or a selected polyol solvent, as described by Myo and Khat-udomkiri [27].

3.1.9 | Identification of Bioactive Chemicals via UHPLC-ESI-QTOF-MS/MS

The phenolic compounds in VT extracts were analyzed using an ultrahigh-performance liquid chromatography (UHPLC) system—specifically, the Agilent 1290 Infinity II coupled with the Agilent 6545 LC-QTOF/MS, as detailed by Khat-Udomkiri and Petsringoen [71].

3.1.10 | Cell Culture

This study assesses the cytotoxicity, cellular antioxidant activity, and wound-healing potential of VT extracts in HaCaT keratinocytes obtained from Thermo Fisher Scientific (Massachusetts, USA). DMEM with 10% fetal calf serum and 1% penicillin/streptomycin was employed to culture the cells at 37°C in a 5% CO_2 incubator. Experiments used passages 8–16 and were performed in three repeats.

3.1.11 | Cytotoxicity Assay

The cell cytotoxicity assay was assessed according to Saelee et al. [72] with modifications. For 24 h, the test substances were incubated with the culture cells. Subsequently, the medium was replaced with 180 μL of fresh serum-free culture medium and 20 μL of PrestoBlue. The mixture was incubated for 3 h. Absorbance was measured at 570 nm using a microplate reader, with 600 nm serving as the reference wavelength. The equation was used to determine cell viability percentage (%):

$$\%\,\text{Cell viability} = \frac{(\text{Absorbance of the sample})}{(\text{Absorbance of control})} \times 100. \quad (5)$$

3.2 | Cytoprotective Efficacy of Extracts Against H_2O_2-Induced Oxidative Stress

According to a modified methodology [73], the cytoprotective effect of extracts was assessed in HaCaT cells subjected to H_2O_2-induced oxidative stress. The culture cells were incubated with test substances for 24 h, followed by treatment with 300 mM hydrogen peroxide for 1 h. The medium was then replaced with 180 μL of fresh serum-free culture medium and 20 μL of PrestoBlue. After a 3-h incubation, absorbance was assessed at 570 nm, utilizing 600 nm as a reference wavelength. The equation was used to determine cell viability percentage (%):

$$\%\,\text{Cell viability} = \frac{(\text{Absorbance of the sample tested with } H_2O_2)}{(\text{Absorbance of control})} \times 100. \quad (6)$$

3.2.1 | Wound-Healing (Scratch) Assay

A wound-healing assay, following a previously established protocol, was conducted to evaluate the extract's ability to promote cell migration [1]. HaCaT cells were seeded into six-well plates at a density of 6×10^5 cells per well and incubated for 24 h at 37°C in a 5% CO_2 incubator. To create a scratch in the adherent cell culture layer, a 200-μL sterile pipette tip was employed. The detached cells were removed by rinsing with D-PBS. The extract was then added, and cells were incubated for an additional 20 h under the same conditions. A positive control was provided by AT at concentrations of 100 and 500 μg/mL. Wound closure was observed using an inverted bright-field microscope with a 4x objective, and image analysis was performed with ImageJ software. Wound coverage was assessed at the initial and final time points from triplicate experiments. The percentage of wound closure was then calculated:

$$\%\,\text{Wound closure} = \frac{((\text{Area of the scratch at initial}) - (\text{Area of the scratch after 20 h}))}{(\text{Area of the scratch at initial})} \times 100. \quad (7)$$

3.3 | Statistical Analysis

Data obtained from the simplex-lattice mixture design and CCD in RSM were analyzed utilizing R software (Version 4.4.1) with the mixexp and rsm packages. A one-sample *t*-test was used to evaluate the differences between the actual and predicted values. Group mean differences were assessed using one-way ANOVA, followed by Fisher's LSD post hoc test. Statistical significance was set at $p < 0.05$.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

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

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. *(Supporting Information)*

Table S1 Expected and observed values of the TPC obtained from a simplex-lattice design.

Table S2 Independent variable levels for central composite design.

Table S3 Design of the central composite with varying responses.

Table S4 Analysis of the model's variances using CCD.

Table S5 Analysis of phenolic constituents in VT leaf extracts using LC-QTOF-MS/MS.

Table S6 Cell migration in a scratch assay over a 20-h period exposure of test substances.

Figure S1. Mass spectrometry total ion chromatograms of VT extracts under positive ionization mode [a]U-BG, [c] M-BG, [e] U-E, and [g] M-E and under negative ionization mode [b]U-BG, [d] M-BG, [f] U-E, and [h] M-E.

Figure S2. MS/MS spectra of Embelin [a], Glucosyringic acid [b], and (R)-Byakangelicin 3-glucoside [c].