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In vitro antioxidant capacity, total phenolic and flavonoid contents, and wound healing potential in *Mangifera indica* L. leaf extracts

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Abstract

Mangifera indica L. is a rich source of biological active compounds, prominently Mangiferin, characterized by its glycosylated xanthone structure. This compound is abundant in various edible sources, such as mango, and exhibits diverse biological activities. Extensive investigation into Mangiferin has highlighted antioxidant, anti-inflammatory, antidiabetic, anticancer, and antimicrobial properties. In this study, Mangiferin extract was prepared through maceration in 80% ethanol, yielding 1,628 g from an initial dry leaf mass of 4,234 g of dried leaf material. The ethanolic extract showed notable torox equivalent antioxidant capacity (TEAC) values for 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays, measured at 0.32 ± 0.03 and 0.56 ± 0.01 mM Trolox equivalents per gram dry material, respectively. The ferric reducing ability power (FRAP) value was determined to be 6.95 ± 0.40 mM $FeSO_4$ per milligram dry material. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays conducted on human adult calcium temperature (HaCaT) cell line indicated that concentrations ranging from 3.90 to 31.25 µg/mL did not significantly impact cell viability. Evaluation of the wound healing of the Mangiferin ethanolic extract demonstrates a consistent decrease in wound area for treated cells compared to untreated controls, with significant decreases observed after 48 hours ($p < 0.05$). These findings support the antioxidant and wound healing properties of the Mangiferin ethanolic extract, underscoring its therapeutic potential in promoting wound repair.

Keywords: *Mangifera indica* L., human keratinocyte, wound healing activity, maceration, antioxidant capacity

1. Introduction

Mangiferin, a xanthone compound, is abundantly found in various parts of the mango tree (*Mangifera indica* L.), including the peel, stalks, leaves, bark, kernel, and stone. Current phytochemical research aims to uncover novel functions of mango leaves, which are rich in antioxidants, phenolic compounds, and flavonoids. These compounds play key roles in preventing oxidative stress-related diseases and aging in humans [1]. Several studies have investigated the antioxidant activities of mango-derived compounds. For instance, Jose *et al*. (2018) demonstrated that mango leaf extract possesses 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and exhibits superoxide dismutase (SOD)-like activity [2]. Additionally, Garcia *et al*. (2002) reported that Mangiferin extracted from stem bark reduces reactive oxygen species (ROS) production by peritoneal macrophages in a mouse model [3]. Coelho *et al*. (2019) investigated the antioxidant potential of mango pulp and peel extracts in methanol, revealing significant antioxidant activity against DPPH and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals [4]. Beyond antioxidant properties, pharmacological studies indicate the wound healing potential of mango (*M. indica* L.). The preparation of extracts from various parts of the mango tree has been shown to alleviate symptoms associated with gastrointestinal diseases, respiratory ailments, urinary tract infections, inflammation, and insect bite irritation. Notably, mango's therapeutic properties

include promoting wound healing, which is particularly beneficial for chronic wounds in diabetic and elderly patients [5, 6, 7].

Mangiferin is a compound present in various parts of the mango tree, with leaves typically exhibiting higher concentrations than other parts. Previous studies have compared Mangiferin content in the leaves of three mango cultivars; Nam Dok Mai, Khiao Sawoei, and Kaew. The results indicated that the Nam Dok Mai cultivar demonstrated the highest Mangiferin concentration [8]. This study investigates the biochemical properties of Mangiferin prepared from the "Nam Dok Mai" variety of mango (*M. indica* L.) using a combination of maceration and partial crystallization techniques in ethanol solvent system. The Mangiferin extract comprehensively evaluated for antioxidant capacity using various methods, including DPPH, ABTS scavenging, and ferric reducing ability power (FRAP) assays. To identify the potential active constituents, total phenolic and flavonoid contents were determined through the Folin-Ciocalteu and aluminum chloride methods, respectively, based on colorimetric principles. Moreover, the *in vitro* wound healing potential of the Mangiferin ethanolic extract was explored using the HaCaT human keratinocyte cell line. To ensure non-cytotoxic effects, cell viability is assessed before evaluating the extract's ability to promote wound healing in the HaCaT cells through the scratch assay technique. This research investigates the antioxidant properties, identifies bioactive compounds, such as phenolic and flavonoid compounds, and assesses the cytotoxic and wound-healing potential of a Mangiferin ethanolic extract. The findings of this study further substantiate the therapeutic efficacy of wound healing. These results may have significant medical implications, potentially leading to the development of effective wound-healing treatments.

2. Materials and methods

2.1 Preparation of Mangiferin ethanolic extract from Mangifera M. indica L. leaves

The preparation of ethanolic extract from *M. indica* L. leaves was modified from the method described by Jutiviboonsuk and Sardsaengjun (2010) [8]. Briefly, *M. indica* L. leaves were washed with water and dried in a hot air oven. The dried leaves were then ground into a find powder combined with 80% ethanol (16 Liters) and subjected to maceration for three days with frequent stirring. After maceration, the liquid extract was separated from the solid material using the cotton filter. The remaining solid residue underwent two additional rounds of maceration in ethanol, and the resulting liquid extracts were combined. The excess solvent was then removed using a rotary evaporator.

The ethanolic extract was further partially purified by the crystallization method [8]. After excess solvent removal, the solid residue was dissolved in 95% ethanol to achieve a final concentration of approximately 330 mg/mL. This solution was continuously stirred at 60 – 70 °C for 2 hours, then filtrated while maintaining this temperature. The resulting precipitate was washed with cold 95% ethanol and dried in a hot air oven to yield ethanolic crystals. These crystals were then solubilized in 95% ethanol within the temperature range of 60 - 70°C. The solution was stored at 4°C for 24 hours, after which the crystals were isolated by filtration followed by thorough drying in a hot air oven. The identification of Mangiferin ethanolic extract was conducted thin-layer chromatography (TLC) and compared with standard Mangiferin, following the method published by Jutiviboonsuk and Sardsaengjun (2010) [8].

2.2 Determination of antioxidant activity by DPPH and ABTS scavenging assays

DPPH solution was prepared in 95% methanol to determine the antioxidant activity of the Mangiferin ethanolic extract in an analytical context. The DPPH concentration was determined by an extinction coefficient of 10,900 $M^{-1}cm^{-1}$ at 515 nm, as reported by Xie and Schaich (2014) [9]. For the DPPH scavenging assay, standard antioxidant agents were employed at varying concentrations 0.63 – 10 µg/mL ascorbate, 3.91 - 125 µg/mL butylated hydroxy toluene (BHT), and 1.56 - 25 µg/mL Trolox. The reaction mixture had a total volume of 1 mL containing 20 µL of either the Mangiferin ethanolic extract or the standard antioxidant agents with 0.1 mM DPPH. The reaction mixture was incubated at room temperature in darkness for 2 hours. Following incubation, absorbance at 515 nm was measured using a UV-VIS spectrophotometer (GENESYS 10S Thermo Fisher Scientific, USA). DPPH solution and 95% methanol were employed as control and blank, respectively.

ABTS scavenging assay was slightly modified from the procedure described by Re et al. (1999) [10]. In this modified procedure, ABTS radical cations ($ABTS^{\bullet+}$) were generated by mixing 7 mm ABTS with 2.45 mM potassium persulfate ($K_2S_2O_8$) at a ratio of 1: 0.5 by volume. The mixture was incubated in the light-protected container at 4°C for 16-20 hours before use. The extinction coefficient of $ABTS^{\bullet+}$ solution was measured in 95% methanol at 734 nm, as reported [10]. To perform the ABTS scavenging assay, standard antioxidant agents were used at varying concentration range of 0.17 - 5 µg/mL ascorbate, 7.81 - 250 µg/mL BHT, and 0.47 - 15 µg/mL Trolox. The reaction mixture had a total volume of 1 mL comprising 20 µL of either Mangiferin ethanolic extract or standard antioxidant agents with 66 µM $ABTS^{\bullet+}$. The reaction was incubated in the dark at room temperature

for 10 minutes. Subsequently, absorbance at 734 nm was measured using a UV-VIS spectrophotometer (GENESYS 10S Thermo Fisher Scientific, USA). The $ABTS^{\bullet+}$ solution and 95% methanol were employed as control and blank, respectively. The DPPH and ABTS scavenging assay were performed as inhibitory percentages using Equation (1).

$$\% \text{ inhibition} = 100 - [(Abs_0 / Abs_1) * 100\%] \quad (1)$$

Where Abs_0 and Abs_1 represent the absorbances of DPPH radical (515 nm) or $ABTS^{\cdot+}$ (734 nm) with or without a dry material (standard antioxidant agents or Mangiferin ethanolic extract), respectively. The inhibitory percentage from "Equation 1" was subsequently used to calculate the half maximum inhibitory concentration (IC_{50}). The IC_{50} value was analyzed and fitted using the non-linear sigmoidal curve equation (dose-response logistic) in Kaleida Graph software (Synergy Software, Reading, PA, USA) according to Equation (2) [11].

$$Y = m_1 + (m_2 - m_1) / (1 + (X / m_3) \wedge m_4 \quad (2)$$

Where m_1 represents the maximum value on the y-axis, m_2 the minimum value on the y-axis, m_3 the X value at the midpoint of the Y range, and m_4 the slope of the curve at the midpoint.

Antioxidant activity was assessed using torox equivalent antioxidant capacity (TEAC) determined by establishing Trolox standard curve, with results subsequently quantified and reported as mM Trolox equivalents (TE) per gram dry material [12].

2.3 FRAP assay

FRAP assay was conducted under a modified procedure as described by Langley-Evands SC [13]. To prepare the FRAP reagent, a solution was prepared by mixing 300 mM acetate buffer pH 3.6, 20 mM ferric chloride ($FeCl_3$), and 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ), adjusting the final volume to 500 mL with 40 mM hydrochloric acid (HCl). These reagents were mixed in a volumetric ratio of 70:15:15. To perform the FRAP assay, standard antioxidant agents were used at varying concentrations of 12.5 - 50 µg/mL ascorbate, 250 - 1,000 µg/mL BHT, and 12.5 - 50 µg/mL Trolox. The assay measures the reduction of the Fe^{3+}-TPTZ complex to Fe^{2+} (ferrous) under acidic conditions. The FRAP reagent was preheated to 37 °C for 5 minutes. The reaction mixture contained 900 µL of preheated FRAP reagent and 100 µL of either the Mangiferin ethanolic extract or the standard antioxidant agents. The reaction mixture was then incubated for 5 minutes, and the absorbance change at 593 nm was measured using a UV-VIS spectrophotometer (GENESYS 10S Thermo Fisher Scientific, USA). Antioxidant activity via FRAP assay was determined based on ferrous sulfate ($FeSO_4 \cdot 7H_2O$) standard curve over a concentration range of 0.01 - 0.1 mM. The FRAP value is reported as mM^{-1} $FeSO_4 \cdot 7H_2O$ per milligram dry material.

2.4 Determination of total phenolic and flavonoid contents

The total phenolic content (TPC) was quantified using a colorimetric method with Folin-Ciocalteu reagent (Bio-Rad, United States), adapted from Ainsworth and Gillespie (2007) [14]. Briefly, a range of gallic acid concentrations in 1.56 - 50 µg/mL mg/mL was prepared to calibrate the assay. The reaction mixture contained 100 µl of either gallic acid (standard) or the Mangiferin ethanolic extract, combined with 10 µL Folin-Ciocalteu reagent. After 5-minute incubation at 25 °C, 500 µl of 1 M Na_2CO_3 was added, and the final volume was adjusted to 1 mL with 95% methanol. The mixture was incubated at 25 °C for 2 hours, and the absorbance at 765 nm was measured using a UV-VIS spectrophotometer (GENESYS 10S Thermo Fisher Scientific, United States). TPC was quantified and reported as mg gallic acid equivalents per gram dry material (mg GAE/g DM).

The TFC was quantified using a colorimetric method based on aluminum chloride ($AlCl_3$), as described by Zhishen *et al*. (1999) [15]. Briefly, a calibration curve was generated using a range of quercetin concentrations (20 - 680 µg/mL) as the standard for flavonoid compounds. Each reaction mixture contained 20 µL of either quercetin or the Mangiferin ethanolic extract, combined with 30 µl of 5% w/v sodium nitrite ($NaNO_2$) and incubated for 5 minutes. Then, 30 µL of 10% w/v $AlCl_3$ and 200 µl of 1 M sodium hydroxide (NaOH) were added. The final volume was adjusted to 1 mL with 95% methanol. Absorbance was measured at 510 nm using a UV-VIS spectrophotometer (GENESYS 10S Thermo Fisher Scientific, USA). TFC was quantified and expressed as milligram quercetin equivalents per gram dry material (mg QE/g DM).

2.5 Cell viability assay of HaCaT cell line (human keratinocyte)

The cell viability assay was performed using the human keratinocyte (HaCaT), kindly provided by Prof. Dr. Praneet Opanasopit from the Faculty of Pharmacy at Silpakorn University, Thailand. The cells were cultured in dulbecco's modified eagle's medium, high glucose media (DMEM-HG) supplemented with 10% fetal bovine serum (FBS) and maintained at 37 °C with 5% CO_2 in a humidified incubator. The Mangiferin ethanolic extract was prepared by dissolving the crystallized powder in dimethyl sulfoxide (DMSO) to achieve a final concentration of 2 µg/mL, and the solution was stored at 4 °C until use. During the cell viability assay, the final concentration of DMSO in each assay was carefully controlled to not exceed 1% to prevent potential DMSO-induced cytotoxicity.

HaCaT cells were seeded in 96-well microplates at a density of 5,000 cells per well and incubated at 37 °C with 5% CO_2 in a humidified incubator for 24 hours before treatment. The cells were then treated with the culture media containing various concentrations of the Mangiferin ethanolic extract, solubilized in DMSO, ranging from 0 - 125 µg/mL. Cell viability assay was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide **(**MTT**)** assay. In this experiment, the solvent control included an additional 1% DMSO in the culture media. Cell viability assay was determined with an MTT assay. After 24-hour treatment, 10 µL of 5 mg/mL MTT solution was added to each well and incubated at 37 °C with 5% CO_2 for 4 hours. Viable cells converted MTT into insoluble formazan crystals, which subsequently solubilized in 200 µl DMSO. Cell viability was quantified measuring the intensity of the solubilized formazan at an absorbance at 540 nm using a microplate reader (Multiskan™, Thermo Fisher Scientific, USA). The percentage of cell survival was calculated relative to a solvent control experiment (1% DMSO without the Mangiferin ethanolic extract in the culture medium). Each experiment was performed triplicate.

2.6 Wound healing assay

The standard *in vitro* technique for investigating collective cell migration in two dimensions is commonly referred to as the wound healing assay. In this study, the wound healing assay was performed on a monolayer of HaCaT cells grown on a plastic substance. This method was adapted from the procedure outline by Jonkman *et al*. (2014) [16]. Briefly, HaCaT cells were maintained at 37 °C with 5% CO_2 in a humidifier incubator for 48 hours. These cells were propagated in DMEM-HG supplemented with 10% FBS and seeded at a density of 2 x 10^5 cells/mL on culture dish. A cell-free gap in a confluent HaCaT cell monolayer was created using the scratch method, where a sterile 200 µl pipette tip was gently used to scratch the cell layer, forming a consistent gap.

To evaluate the wound healing response, HaCaT cells were treated with the Mangiferin ethanolic extract at concentrations ranging from 3.75 - 30 mg/mL. This concentration range was selected based on preliminary cell viability assay results, which confirmed the absence of cytotoxicity. Images was acquired using an inverted light microscope (Olympus CKX31 Inverted Phase Contrast Microscope, Japan) and the wound area was quantified in square micrometer (μm^2) using automated software, specifically "ZEN Microscopy version 3.6 Blue edition (ZEISS, Germany)". The wound healing response in HaCaT cells following treatment with the Mangiferin ethanolic extract was assessed at 24- and 48-hour intervals, compared to a negative control that received no Mangiferin ethanolic extract. The wound healing experiments were independently repeated three times to ensure the statistical significance of the results.

2.7 Statistical analysis

The results of three independent experiments are presented as means $\pm$ standard deviation. Statistical significance was determined using One-way ANOVA, with a *p-value* of less than 0.05 considered significant. All statistical analyses were performed using version 15 of the Statistical Package for the Social Sciences (SPSS) software.

3. Results and discussion

3.1 Mangiferin preparation and antioxidant assay

The Mangiferin ethanolic extract was prepared through maceration in 80% ethanol, yielding 1,628 g from a raw material dry weight of 4,234 g, resulting in a recovery yield of 38.44%. The ethanolic extract was subsequently partially purified by crystallization and subjected to a comprehensive set of assays, including the evaluation of antioxidant capacity, quantification of total phenolic and flavonoid contents, *in vitro* cytotoxicity assay, and determination of wound healing potential. This multifaceted analysis was conducted to elucidate the biochemical profile of the Mangiferin ethanolic extract and to explore its potential therapeutic applications (Table 1).

Table 1 Antioxidant activity, total phenolic and flavonoid contents in the Mangiferin ethanolic extract from *M. indica* L.

Sample	DPPH scavenging assay		ABTS scavenging assay		FRAP	Total	Total flavonoid[§]
	IC_{50} (μg/mL)	TEAC*	IC_{50} (μg/mL)	TEAC*	value**	phenolic[‡] mg GAE/g DM	mg QE/g DM
Mangiferin ethanolic extract	77.93 ± 0.18	0.32 ± 0.03	29.75 ± 0.55	0.56 ± 0.01	6.95 ± 0.40	1.69 ± 0.04	459.13 ± 39.06
Standard antioxidant agent							
Ascorbic acid	5.28 ± 0.12	5.13 ± 0.08	2.76 ± 0.06	6.34 ± 0.25	99.13 ± 8.02		
BHT	18.10 ± 3.30	1.92 ± 0.15	8.99 ± 0.55	0.99 ± 0.01	18.09 + 1.34		

Data is showed as mean ± standard deviation (SD) with triplicated values.
* TEAC can be calculated from the Trolox standard curve and expressed as mM Trolox Equivalents per gram dry material.
**FRAP value is expressed as mM $FeSO_4 \cdot 7H_2O$ per milligram dry material.
[‡]Total phenolic content is expressed as mg Gallic acid Equivalent (GAE) per gram dry material.
[§]Total flavonoid content is expressed as mg of Quercetin Equivalent (QE) per gram dry material.
N/A - Trolox serves as a standard calibrant for the determination of TEAC value.

3.2 Antioxidant capacity, total phenolic and flavonoid contents in Mangiferin ethanolic extract

The conventional method for accurately measuring antioxidant activity challenging to establish [17, 18]. This inherent complexity required the use of multiple methods to comprehensively evaluate the antioxidant capacity in plant extracts. The methodologies are employed to evaluate the antioxidant potential of plant extracts. Among these, the DPPH, ABTS, and FRAP assays are commonly used procedures for assessing antioxidant capacity. The DPPH method is widely employed for determining antioxidant capacity in natural plant extracts due to its stable free radical nature, suitability for laboratory conditions, and ease of time-dependent monitoring. The ABTS assay measures the relative ability of antioxidants to scavenge radicals in both aqueous and organic solvent phases. The FRAP assay is relatively easy to perform and can be completed within a short time frame, and it is less susceptible to interference from other compounds present in plant extracts. Each antioxidant assay utilizes a different mechanism of action, enabling the assessment of various types of antioxidants. This approach facilitates the identification of specific compounds responsible for antioxidant capacity and the elucidation of their underlying mechanisms. By combining these assays, a more accurate and comprehensive evaluation of the antioxidant potential of plant extracts can be achieved. This information is crucial for the development of novel natural antioxidant products and a deeper understanding of their modes of action. In this study, the antioxidant capacity of the Mangiferin ethanolic extract was evaluated using DPPH and ABTS scavenging assays, with results reported as IC_{50} and TEAC values. Additionally, the FRAP method was employed to further evaluate antioxidant capacity. A comparative analysis with standard antioxidant agents such as BHT and ascorbic acid was conducted to elucidate the antioxidant potential of the Mangiferin ethanolic extract.

As shown in Table 1, the Mangiferin ethanolic extract exhibited notable antioxidant capacity, as indicated by DPPH scavenging assay with IC_{50} values of 77.93 ± 0.18 μg/mL and TEAC value of 0.32 ± 0.03 mM TE per gram dry material. Similarly, ABTS scavenging assay demonstrated an IC_{50} value of 29.75 ± 0.55 μg/mL and TEAC value of 0.56 ± 0.01 mM TE per gram dry material. This potency was further confirmed by the FRAP value of 6.95 ± 0.40 mM^{-1} $FeSO_4 \cdot 7H_2O$ per mg dry material. Comparing the antioxidant capacity of the Mangiferin ethanolic extract and standard antioxidant agents, as indicated by TEAC and FRAP values, suggests that the scavenging ability of the Mangiferin ethanolic extract against DPPH and ABTS radicals is comparable to that of BHT (Figure 1), as illustrated in Figure 1(A) (DPPH scavenging assay), Figure 1(B) (ABTS scavenging assay), and Figure 1(C) (FRAP assay). The selection of the solvent extraction system plays a significant role in the evaluation of antioxidant activity, primarily due to its impact on the solubility and compatibility of the extract with analytical techniques. A study by Kawpoomhae *et al*. (2010) investigated the antioxidant capacity of fresh mango leaf extracts using various solvent extraction systems such as methanol, water, and chloroform, reporting the IC_{50} of DPPH values of 6.18 ± 0.15, 5.57 ± 0.18, and 72.40 ± 3.24 μg/mL respectively, and ABTS IC_{50} values of 1.33 ± 0.13, 2.96 ± 0.05, and 6.56 ± 0.49 μg/mL, respectively [19]. The IC_{50} value serves as an indication of the relative antioxidant capacity of analytical samples. It is defined as the concentration necessary to inhibit 50% of the radical agent (DPPH or ABTS radical). The difference in IC_{50} values may occur from variations in the extraction methods employed in the current and previous studies. High-temperature processing during extraction

can potentially lead to the degradation of certain bioactive compounds, consequently impacting the overall antioxidant capacity.

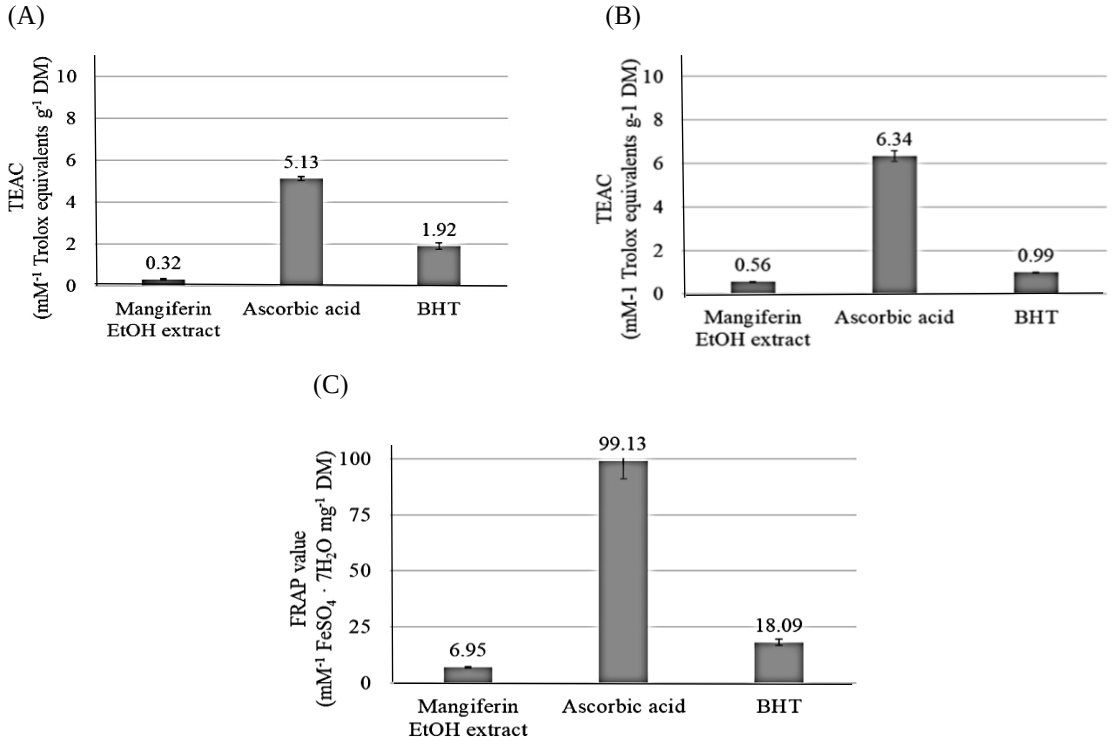

Figure 1 Comparison of the antioxidant capacity (TEAC and FRAP values) between the Mangiferin ethanolic extract and standard antioxidant agents (ascorbic acid and BHT); Figure 1(A): DPPH scavenging assay; Figure 1(B): ABTS scavenging assay; Figure 1(C): FRAP assay. Data are presented as mean ± standard deviation (SD) with triplicated measurements.

Phenolic and flavonoid compounds are recognized as the ubiquitous secondary metabolites in plants, renowned for their broad spectrum of biochemical activities, including antioxidant, anticancer, and antimicrobial [20, 21]. The total phenolic contents were determined using Folin-Ciocalteu method, based on a standard curve of gallic acid, resulting in a value of 1.69 ± 0.04 mg GAE/g DM (Table 1). Similarly, the total flavonoid contents were determined using a colorimetric method (aluminum chloride), based on a standard curve of quercetin, with a resulting value of 459.13 ± 39.06 mg QE/g DM (Table 1).

These findings align with prior studies, which reported a concentration-dependent increase in DPPH radical scavenging activity [22] and a linear relationship between polyphenol concentration and antioxidant activity [23] The enhanced antioxidant activity of mangiferin, a polyaromatic polyphenolic compound, is attributed to mechanisms like resonance energy transfer, phenoxy radical delocalization, O-H bond dissociation, and steric hindrance [24] [25]. Notably, the purity and concentration of mangiferin play a critical role in its antioxidant capacity.

3.3 The effect of Mangiferin ethanolic extract concentration on HaCaT cell viability

The cytotoxicity of the Mangiferin ethanolic extract on the HaCaT cell line human keratinocyte, Figure 2 was evaluated using the MTT assay for 24 hours at concentrations ranging from 3.9 to 125 µg/mL. A solvent control containing 1% DMSO in the culture medium, without the Mangiferin ethanolic extract, was included in the experiment. As shown in Figure 3, the results indicate that the Mangiferin ethanolic extract exhibits no cytotoxic effects within the validated concentration range of 3.90 - 31.25 µg/mL. This concentration range was subsequently used to assess the wound healing potential of the Mangiferin ethanolic extract in the HaCaT cell line.

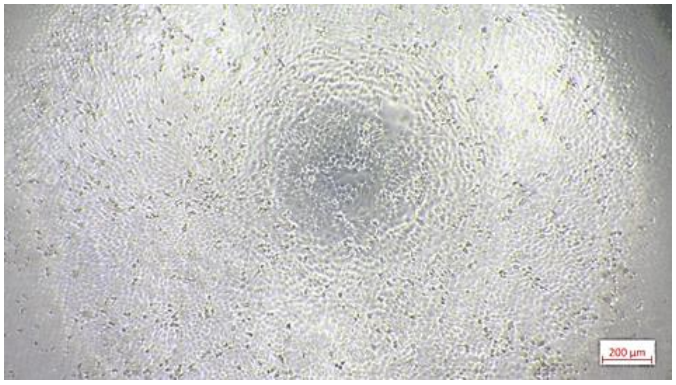

Figure 2 HaCaT cell (keratinocyte) under 4X magnification of an inverted microscope.

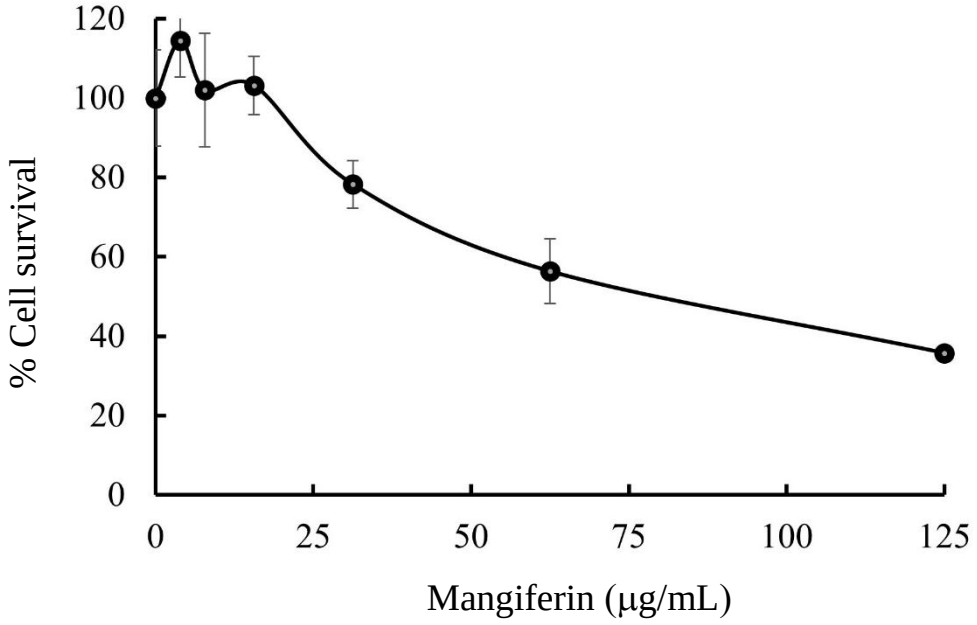

Figure 3 Percent cell survival of HaCaT cell line after 24 hours incubation with 3.9 - 125 µg/mL Mangiferin ethanolic extract. Data are presented as mean ± standard deviation (SD) from triplicate measurement.

3.4 The effect of Mangiferin ethanolic extract on wound healing activity

This study investigated the wound healing potential of Mangiferin ethanolic extract on HaCaT cell line using the scratch method. Treatment with a concentration range of 0-30 µg/mL l was applied, and wound closure was monitored for 24 and 48 hours. A notable reduction in wound area was observed, documented using an Inverted Light and a Zeiss camera. The results suggest that the Mangiferin ethanolic extract promotes migration and proliferation of HaCat cell as evaluated by creating a scratch wound on the cell monolayer and quantifying its closure over 24 and 48 hours (Figure 4).

The assessment involved comparing the initial wound area on Day 0 (defined as 100% wound area) with subsequent measurements on Day 1 (24 hours) and 2 (48 hours). The results revealed a consistent decrease in wound area in cells treated with the Mangiferin ethanolic extract compared to untreated controls (Figure 5), with a particularly significant decrease observed on Day 2 ($p<0.05$, One-way ANOVA). Interestingly, no significant variance in wound area reduction was observed across different concentrations of the Mangiferin ethanolic extract. The antioxidant assay showed a dose-dependent effect for Mangiferin; however, in the wound healing assay, keratinocytes treated with Mangiferin showed no correlation with wound healing outcomes. This suggests that the extract's wound healing effects may arise from compounds other than antioxidants.

The high bioavailability of Mangiferin makes it a promising candidate for potential biomedical applications, such as wound healing products. *In vitro* experiments by Allaw *et al*. (2020) reported that a Mangiferin formulation promoted the fibroblasts migration and proliferation, assessed by creating a linear scratch wound and quantifying its closure over 48 hours [26]. Additionally, Daud *et al.* (2010) demonstrated that Mangiferin extracts increased endothelial cell migration, potentially promoting new blood vessel formation during wound healing [27]. Further, Espinosa-Espinosa L *et al.* (2022) investigated wound healing in a murine model using the incision technique, implying that a methanolic mango peel extract exhibited healing efficacy, evidenced by wound contraction and histological analysis [7]. Our findings will contribute to the expanding range of evidence supporting the wound healing properties of the Mangiferin ethanolic extract, highlighting its potential therapeutic value. In diabetic wound, Mangiferin significantly reduced wound size, increased surrounding skin thickness, and upregulated growth factors such as EGF, VEGF, and TGF-β while downregulating inflammatory markers like TNFα and NF-κB [28]. In immunocompromised models, mangiferin promoted angiogenesis, reduced inflammation, and enhanced collagen and granulation tissue formation via VEGF and COX-2 signaling pathways [29].

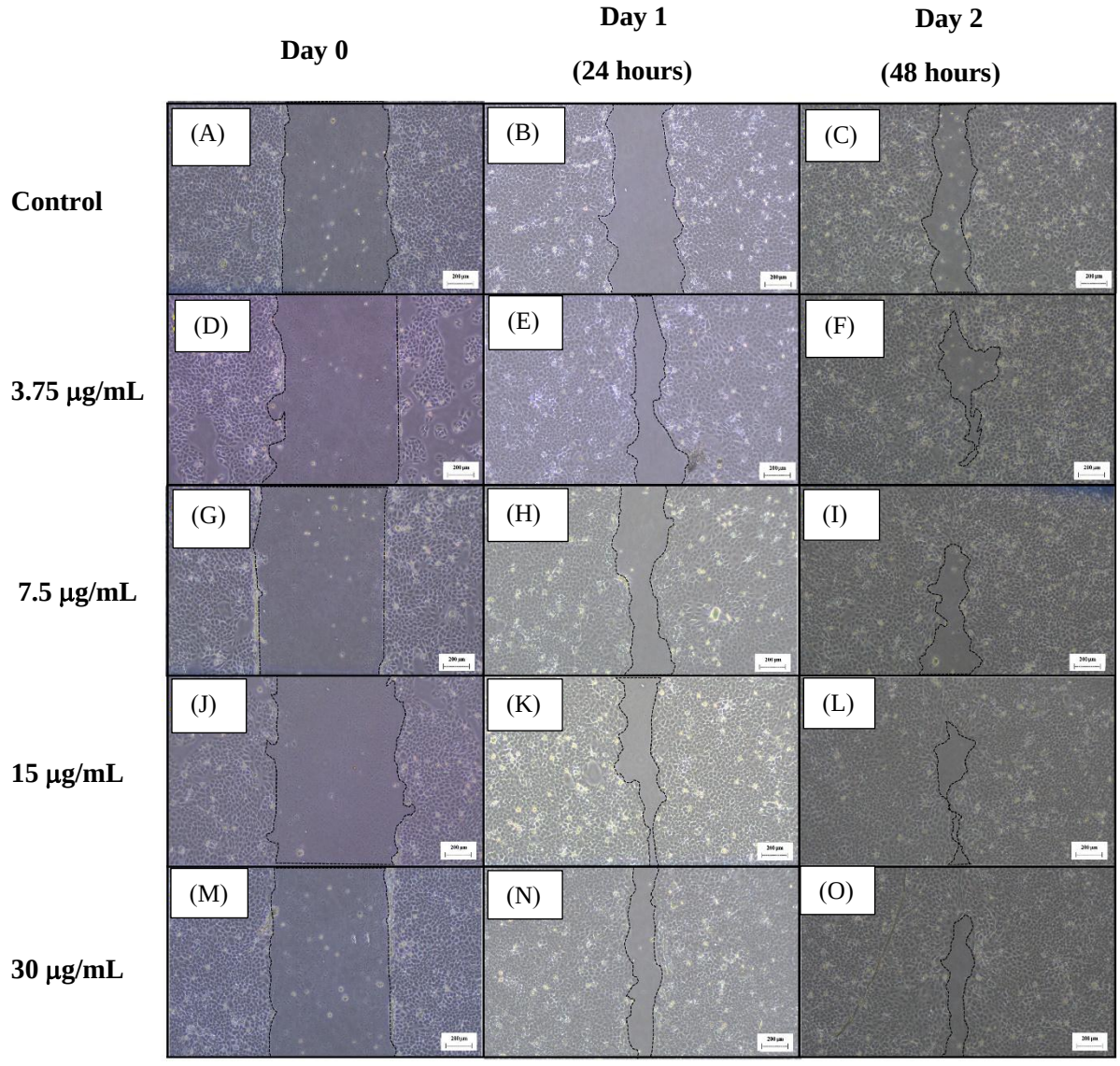

Figure 4 Wound area of HaCaT cell line observed under inverted light microscope following treatment with the Mangiferin ethanolic extract at concentration of 3.75 – 30 µg/mL on Day 0, Day 1 (24 hours incubation) and Day 2 (48 hours incubation); Figure 5(A)-5(C): control; Mangiferin ethanolic extract treatment in Figure 5(D)-5(F): 3.75 µg/mL; Figure 5(G)-5(I): 7.5 µg/mL; Figure 5J-5L: 15 µg/mL; Figure 5(M)-5(O): 30 µg/mL.

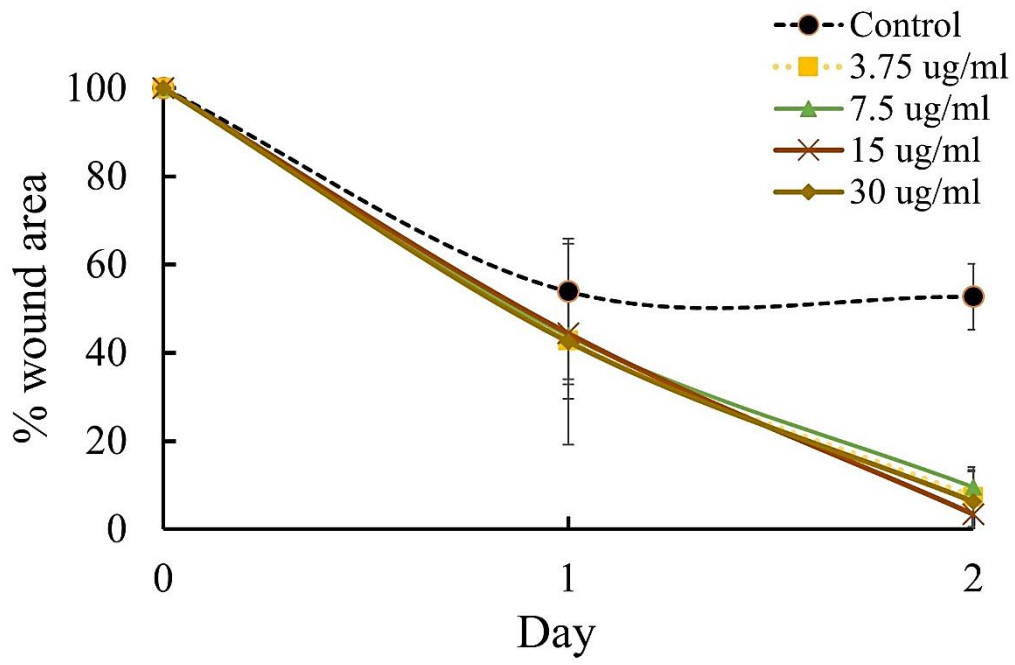

Figure 5 Percentage of wound area of HaCaT cell line after 24 hours (Day 1) and 48 hours (2 Day) of incubation with 3.75 - 30 µg/mL Mangiferin ethanolic extract. Data are presented as mean ± standard deviation (SD) from triplicate measurements.

4. Conclusions

Mangiferin, a natural glucoxanthone, is a prominent bioactive compound found in various parts of Mangifera indica, including its leaves, bark, and peels, as well as other plant species [30]. To increase the water solubility, it has been shown to significantly enhance its antioxidant properties [31]. In the present study, the partial purification of Mangiferin extract was achieved through maceration and crystallization under ethanol extraction system. The extract exhibited strong antioxidant activity, as demonstrated by DPPH, ABTS, and FRAP assays, which may be attributed to its free radical scavenging properties. Quantification of its total phenolic and flavonoid content, using the Folin-Ciocalteu and aluminum chloride methods, highlighted its potential nutritional and therapeutic value.

To assess the wound-healing potential of Mangiferin ethanolic extract, HaCaT keratinocyte cells were subjected to a scratch assay. This methodology assay involves creating a scratch wound on a cell monolayer and monitoring its closure over time. Treatment with Mangiferin ethanolic extract resulted in a significant reduction in wound area, particularly after 48 hours. Importantly, MTT assays confirmed that the concentrations used in these experiments were non-toxic to the cells. The findings of this study demonstrate that Mangiferin ethanolic extract promotes wound healing by reducing inflammation and oxidative stress, as evidenced by the significant reduction in wound area and increased cell proliferation.

Mangiferin has also shown promise as a valuable nutraceutical ingredient. For example, hydrogel formulations incorporating Mangiferin and diclofenac sodium have demonstrated enhanced antibacterial, antioxidant, and anti-inflammatory properties, while simultaneously promoting angiogenesis and accelerating wound healing in diabetic wounds [32]. Furthermore, Mangiferin nanoparticles have been successfully incorporated into dairy beverages, resulting in functional foods with elevated antioxidant activity, reduced lipid and protein oxidation, and lower glycemic indices [33]. Similarly, Mangiferin-enriched milk beverages have exhibited improved antioxidant properties and enhanced sensory qualities, such as increased sourness, making them attractive options for innovative functional food formulations [34].

While the *in vitro* results presented in this study are promising, further clinical investigations are necessary to validate the efficacy of Mangiferin ethanolic extract in human subjects. Additionally, optimizing delivery mechanisms for therapeutic applications in wound care and functional foods remains a crucial area of future research.

5. Ethical approval

The research was approved the ethical from the Ethics Committee of Huachiew Chalermprakiet University (Certificate of Exemption no. 1225/2565).

6. Conflicts of interest

The authors declare that there are no conflicts of interest in this research.

7. References

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