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Quantification of Mangiferin from the Bioactive Fraction of Mango Leaves (*Mangifera indica* L.) and Evaluation of Wound-Healing Potential

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ABSTRACT

Burns refer to damage to the skin's surface caused by exposure to high temperatures, which can be due to factors such as oil, water, electricity, fire, sun exposure, and chemicals. Prompt and appropriate treatment is essential to prevent undesirable consequences. Thus, this study aimed to quantify mangiferin, a potential treatment for burns, in the bioactive fraction of mango leaves (Mangifera indica L.) and evaluate its effectiveness in healing burns.The methods employed included thin-layer chromatography (TLC)-densitometry with validation measures, including linearity, detection and quantification limits (LoD and LoQ), precision, accuracy, and quantification. The bioactive fraction was formulated in membranes at concentrations of 5%, 10%, and 15%. These membranes were applied to rabbits previously subjected to six wound burns, and the healing progress was monitored by measuring burn diameter using a vernier caliper every 3 days for a total of 21 days. Mangiferin, the active compound, was detected at a wavelength of 257 nm. Test results yielded a linearity equation, y = 76496x + 2935.7, with a correlation coefficient value of 0.9957, a detection limit of 2.01 µg/mL, a quantification limit of 6.07 µg/mL, a coefficient of variation ranging from 0.59% to 3.33%, and an accuracy range of 99.18% to 100.9%, with mangiferin levels at 208.31 µg/mL. The membrane preparations of the bioactive mangiferin fraction were evaluated on second-degree burns in rabbits, with concentrations of 10% and 15% showing the most effectiveness.

Keywords: bioactive fraction, mangiferin, burns, membrane, quantification.

1. INTRODUCTION

Burns represent a global health challenge with high mortality and morbidity rates, resulting in a minimum of 180,000 deaths annually (1). Furthermore, more than 96% of burn cases occur in low- and middle-income countries (1). According to data from the Health Ministry of Indonesia (2008), the prevalence of burns was reported at 2.2%, contributing to approximately 40% of all deaths (2). Burns have the potential to damage not only the skin but also other critical tissues, including blood vessels, nerves, tendons, and bones (3). They also significantly elevate the risk of infection, which is the primary cause of death in 61% of burn patients (4). This underscores the importance of effectively controlling bacterial infections during burn treatment to substantially reduce mortality. Aerobic bacteria, including Pseudomonas aeruginosa, Staphylococcus aureus, Acinetobacter baumannii, and Klebsiella pneumoniae, are common culprits in burn-related infections (5). Previous studies have revealed the existence of antibiotic-resistant bacteria, such as methicillin-resistant S. aureus (MRSA) (5). Consequently, it is imperative to administer appropriate treatments to burn patients to prevent potentially fatal bacterial infections.

In the exploration of sustainable Sumatran medicinal

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plants and the search for alternative medicines (6, 7), mango leaves (Mangifera indica Linn.) have been reported to have the potential to treat burns. Mango is a tropical plant, and its production volume increases every year. In 2016, Padang City produced 358 tons of mangos, and this volume increased to 1655 tons in 2020 (8); however, the community primarily utilizes only the fruit. Several studies have revealed that the ethanol extract of mango leaves has efficacy as an antifungal, anti-inflammatory, anticancer, antioxidant, and antimicrobial analgesic agent (9-12). The antibacterial activity of mango leaf extract can inhibit the growth of various bacteria, including Staphylococcus aureus, Streptococcus pyogenes, Streptococcus pneumoniae, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Salmonella typhi, and Shigella flexneri, at concentrations ranging from 150 mg/ml to 250 mg/ml, with the effect improving as the concentration increases[13].

The efficacy of mango leaf ethanol extract as an anti-inflammatory, antioxidant, and antimicrobial agent makes it a valuable candidate for burn therapy. From a chemical perspective, it contains various secondary metabolites, with mangiferin being the primary constituent. The structural formula of mangiferin is presented in Figure 1. Several studies have shown that this compound is responsible for the aforementioned pharmacological effects (14). Therefore, the objective of this study is to determine the levels of mangiferin in the bioactive fraction of mango leaves and evaluate its effectiveness in healing burns in rabbits.

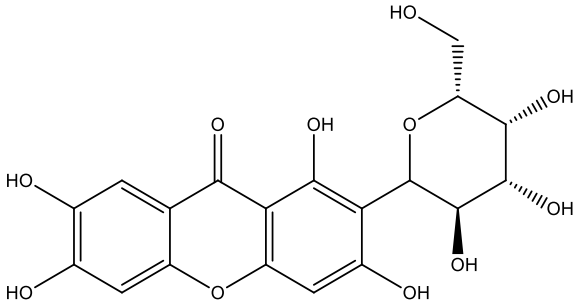

Figure 1. Mangiferin

2. MATERIALS AND METHODS

2.1. Plant material

Mango leaves were collected in 2018 in Padang, West Sumatra, Indonesia. The leaves were identified and authenticated by Dr. Nurainas at the Herbarium Laboratory of the Department of Biology, Faculty of Mathematics and Natural Sciences, Andalas University, under specimen number 460.

2.2. Instrumentations, Reagents and materials

All chemicals and reagents used were of analytical grade, including methanol (Merck®), ethyl acetate (Merck®), n-hexane (Merck®), formic acid (Merck®), silica gel PF 60 (Merck®), glycerin (Merck®), PVA (Merck®), nipagin (Merck®), nipasol (Merck®), and the reference compound mangiferin (phytopure). The equipment included a UV-Vis spectrophotometer (Shimadzu® UV-1700 PharmaSpec) and a **thin-layer chromatography** (TLC) scanner instrument (Camag®).

2.3. Sample Fractionation

The mango leaves were dried, chopped, and mashed to yield a net weight of 1 kg. Extraction was carried out using the maceration method with methanol as the solvent, involving two immersions for 3 × 24 hours each. During extraction, the mixture was periodically stirred, and the mango leaf powder-to-solvent ratio was 1:20. In the second maceration step, the ratio was adjusted to 1:10. Subsequently,

the extracted liquid was concentrated under vacuum to yield a viscous extract. To further process the extract, it was defatted using the fractionation method with n-hexane, resulting in hexane and methanol fractions. Each fraction was monitored using TLC with an ethyl acetate eluent mixture of formic acid and water (36:6:4) and then compared to pure mangiferin compounds. The methanol fraction was subjected to separation via column chromatography using a step gradient polarity involving n-hexane-ethyl acetate (100:0 → 0:100) and ethyl acetate-methanol (100:0 → 0:100). Each subfraction was re-monitored by TLC and compared to mangiferin to determine the presence of these compounds.

2.4. Quantification and Validation of Mangiferin in the Bioactive Fraction by TLC

The TLC plate used was composed of silica gel 60 F254 with dimensions of 20 × 10 cm. The mobile phase consisted of ethyl acetate: formic acid: water (36:6:4), and the chromatography was conducted in a saturated chamber for approximately 45 minutes. Subsequently, the plate was allowed to air-dry and then quantified using the Camag TLC scanner 4 at a wavelength of 257 nm. The obtained results were analyzed with the winCATS application (version 1.4.7), which generated a linear calibration plot based on the standard regression equation[15].

The TLC-densitometry method was validated using several parameters as described in reference [16,17]:

a) Linearity

Linearity was assessed through data analysis using five standard solution concentrations: 0.05, 0.1, 0.2, 0.3, and ***0.4 mg/mL. Each solution (5 µL) was applied to the same*** plate and eluted using a mobile phase with an ethyl acetate: formic acid: water ratio of 36:6:4, followed by scanning using a TLC scanner at a wavelength of 257 nm.

b) Determination of Limit of Detection (LoD) and Limit of Quantification (LoQ)

The detection and quantification limits were utilized to assess the method's sensitivity. LoD and LoQ values were determined based on linear equations derived from the calibration curve, which was constructed using solutions with concentrations of 0.05, 0.1, 0.2, 0.3, and 0.4 mg/mL. Additionally, the solutions were eluted using the mobile phase ethyl acetate: formic acid: water (36:6:4) and evaluated with a densitometer to calculate the standard deviation (SD) value.

$$LoD = \frac{3.3\ x\ SD}{Slope}$$

$$LoQ = \frac{10\ x\ SD}{Slope}$$

c) Precision

Precision was determined using three concentrations: 0.06, 0.12, and 0.2 mg/mL. The elution process with ethyl acetate: formic acid: water (36:6:4) as the mobile phase was repeated three times. Subsequently, the solutions were scanned using a TLC scanner, and the average standard deviation (SD) and percentage coefficient of variation (CV) were calculated.

$$\%CV = \frac{SD}{AUC} \times 100\%$$

d) Accuracy

Accuracy was assessed as a validation parameter to determine the % recovery, which fell within the range of 98–102%. ***This was achieved by adding 60, 120, and 200*** µg/mL to the samples. The elution process employed an ethyl acetate: formic acid: water ratio of 36:6:4 as the mobile phase. After elution, the mixture was scanned using a TLC scanner, and the percentage recovery value was calculated.

e) Quantification of mangiferin

The mangiferin bioactive fraction was prepared at a concentration of 10 mg in 10 mL. Elucidation was conducted using an ethyl acetate: formic acid: water ratio of 36:6:4 as the mobile phase. The area under the curve (AUC) value was obtained using a TLC scanner, and the mangiferin levels in the bioactive fraction were calculated using a linear regression equation.

2.5. Mangiferin Bioactive Fraction Membrane Formulation

The membrane formulation included various additional substances, such as polyvinyl alcohol (PVA), glycerin, nipagin, nipasol, and sterile water as a solvent. Membranes containing the mangiferin bioactive fraction were formulated at different concentrations, namely 5%, 10%, and 15%, to investigate whether its healing activity exhibited a dose-dependent effect on burn wounds.

2.6. Experimental Animal Protocol

This study received approval from the ethics committee of the Faculty of Medicine, Andalas University, under reference number 302/KEP/FK/2019. Four adult male white rabbits (Oryctolagus cuniculus) with an average weight of 2 kg were housed in the Sumatran Biota Laboratory in individual cages maintained at a room temperature of 26 ± 1°C. They had access to a regular supply of food and water. The rabbits were utilized for experiments 7 days after their arrival at the animal facility. The study adhered to the ARRIVE (Animals in Research: Reporting in Vivo Experiments) criteria for animal experiments.

2.7. Evaluating the Mangiferin Bioactive Fraction Membrane on Experimental Animals

In this study, four adult male white rabbits were included, and they were subjected to six different treatments: no treatment (negative control), treatment with Bioplacenton® (positive control), treatment with a membrane base devoid of the mangiferin bioactive fraction, and treatment with membranes containing 5%, 10%, and 15% mangiferin bioactive fractions. Prior to treatment, the rabbits underwent burns induced by applying hot metal with a diameter of 20 mm. Subsequently, treatments were administered 24 hours after the burns, and the rabbits were observed for a period of **21 days**.

2.8. Data Analysis

The average wound diameter was measured in the vertical, horizontal, and diagonal directions. The percentage of healing was calculated using the following formula:

$$\frac{d_1^2 - d_2^2}{d_1} \times 100\%$$

Note:

d1 = the diameter a day after making the wound (mm)

d2 = the diameter of the wound on the day of observation (mm)

The assessment of wound healing percentage was conducted from the 1st day when the test material was administered up to the 21st day. Data analysis was performed using the two-way analysis of variance (ANOVA) method. A post-hoc Duncan test was employed to determine the impact of the membrane preparations on the percentage of burn wound healing within each group. Results were deemed statistically significant if the p-value was less than 0.05.

3 RESULTS AND DISCUSSION

3.1. Sample Fraction

A total of 1 kg of mango leaves was extracted using methanol as a solvent, with a 6.9% yield. However, the value obtained for the methanolic extract was 16.81%[18]. This was caused by several factors, including altitude, temperature, soil type, and other environmental factors. It was then defatted with n-hexane as a solvent to separate the non-polar phase and produce a methanol extract and an n-hexane fraction from the mango leaf extract. The monitoring of mangiferin in each fraction was carried out via TLC with a mobile phase of ethyl acetate: formic acid: water (36:6:4 v/v) to obtain an Rf value of 0.56, as shown in Figure 2. A previous study used a mobile phase comprising ethyl acetate: distilled water: formic acid (8.5:1.5:1) with an Rf value of 0.66[19]. The production of the methanol fraction from the mango leaves was followed by subfraction separation using vacuum column chromatography with a solvent mixture of n-hexane, ethyl acetate, and methanol, adjusted based on polarity levels. Each fraction was monitored using TLC, resulting in the isolation of 44.9 g of a 100% methanol fraction containing mangiferin

compounds. This product was subsequently formulated into a membrane preparation and tested on experimental animals.

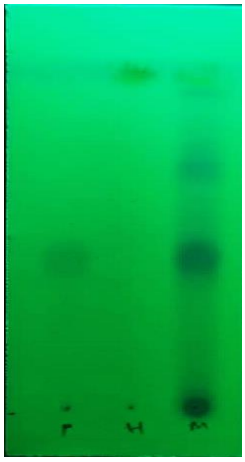

Figure 2. TLC profile of mangiferin with *n*-hexane and methanol extracts with ethyl acetate: formic acid: water (36:6:4) as the eluent, under UV λ_{254} nm (P = pure mangiferin, H = *n*-hexane extract, M = methanol extract)

3.2. Quantification and Validation of Mangiferin in the Bioactive Fraction by TLC-Densitometry

Quantitative analysis is fundamental for providing information about the composition and concentration of secondary metabolites in natural ingredients responsible for specific pharmacological activities. Several analytical methods can be used for quantification, but TLC-densitometry is accurate, simple, and straightforward.[19]

The selective wavelength of mangiferin was obtained at 257 nm, while a wavelength of 258 nm was recorded in another study [20]. The selective wavelength is an identification parameter for mangiferin compounds. The mobile phase used was ethyl acetate: water: formic acid in the ratio of 36:6:4.

3.3. Validation of the Mangiferin Bioactive Fraction

a) Linearity

The linearity analysis was conducted by eluting five concentrations of standard solutions (0.05, 0.1, 0.2, 0.3, and 0.4 mg/mL) on a silica gel 60 F254 TLC plate, followed by the measurement of the area. The calculation yielded the equation y = 76496x + 2935.7 with a correlation coefficient of 0.9957, which falls into the 'fairly good' category, as shown in Figure 3. In contrast, another study obtained a different equation, y = 17,7845x + 194,030, with an R value of 0.9997[19].

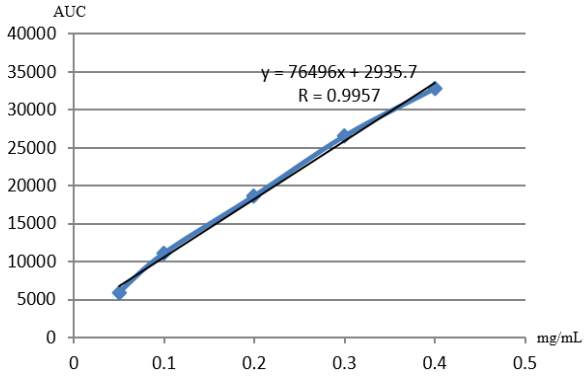

Figure 3. Mangiferin calibration curve

b) LoD and LoQ

The LoD and LoQ values in this study were 2.01 µg/spot and 6.07 µg/spot, respectively. A previous study reported values of 99 ng/spot and 329.8 ng/spot, respectively[19].

c) Precision

The accuracy of this study was analyzed based on the coefficient of variation (CV), which ranged from 0.59% to 3.33%. In another study, the CV value ranged from 0.12% to 0.91%, falling within the required % CV values field, namely % CV < 5%[16,19].

Table 1. Accuracy Test Results

Rate (mg/ml)	SD	% CV
0.06	46.46	0.59
0.12	54.80	1.18
0.2	77.57	3.33

d) Accuracy

The accuracy test was used to determine the proximity of the percentage obtained from the analysis to the actual content of mangiferin. The resulting value was 100.1 ± 0.49% (w/w), falling within the required range of 80–120%[16].

Table 2. Accuracy Value

Actual rate (µg/mL)	Rate earned (µg/mL)	% Recovery
60	60.56	100.9
120	119.02	99.18
200	200.42	100.21

e) Quantification of mangiferin

The quantification of mangiferin in the extract resulted in a value of 208.31 µg/mL, with a yield of 0.94%.

3.4. Mangiferin Bioactive Fraction Membrane Formulation

The evaluation of the mangiferin bioactive fraction membrane preparation formula included visual testing on a white background for colored membranes and on black for colorless ones, as well as testing for homogeneity and thickness. These tests were conducted from the initial gel preparation stage until the formation of the membrane preparation[21].

Based on the results obtained, all membranes received a (++) rating due to their translucent appearance when observed against a white background. Appearance was categorized as follows: (+) cloudy, (++) translucent, and (+++) transparent. The base formula membrane was clear, while the formula membrane containing the bioactive extract exhibited a brown color that intensified with increasing concentrations of the mangiferin bioactive fraction, resulting in a brownish appearance (Fig. 4). Additionally, no lumps were formed, and the preparations were homogeneous[22,23].

The thickness test of the mangiferin bioactive fraction membrane aimed to assess the uniformity of thickness, which is indicative of homogeneity when poured into the mold. Non-uniformity in the material can affect the product's performance. One membrane was tested by measuring it at three different points with a screw micrometer. The examination revealed that the product had an average thickness of 0.19 ± 0.025 mm. Additionally, the results demonstrated that the membrane thickness increased with the concentration of the bioactive fraction of mangiferin. A test for the presence of air bubbles was also conducted, yielding positive results for all products[21,24].

Figure 4. Mangiferin Bioactive Fraction Membrane with concentration: a. 0%, b. 5%, c. 10%, and d. 15%

3.5. Evaluating Membrane Activity on Burns

The activity of membrane preparations containing the bioactive fraction of mangiferin was tested on white male rabbits (Oryctolagus cuniculus). This study aimed to evaluate

the membranes' ability to accelerate the healing of burns on the rabbits' back skin, using concentrations of 5%, 10%, and 15%. Their effectiveness was also compared with that of the standard Bioplacenton, known for its antibacterial activity in wound healing. The study assessed the burn diameter[25].

Burn healing is a complex physiological process in which damaged skin tissue returns to its normal anatomical state following thermal injury. During the healing process, keratinocytes and epidermal cells from the periphery of the damaged tissue proliferate, reducing the area of the injury. In this study, burn healing was assessed through observations after superficial burns were induced in four male rabbits, with an average diameter of 21.49 mm. The membrane preparation was administered to the test animals once every three days, while Bioplacenton was administered once daily[26,27].

Observation of the wound area in all groups over the 21-day period revealed changes in wound size. The period from days 0 to 6 corresponded to the inflammatory phase, while days 6 to 21 marked the proliferation stage, involving the repair of injured tissue[27-29].

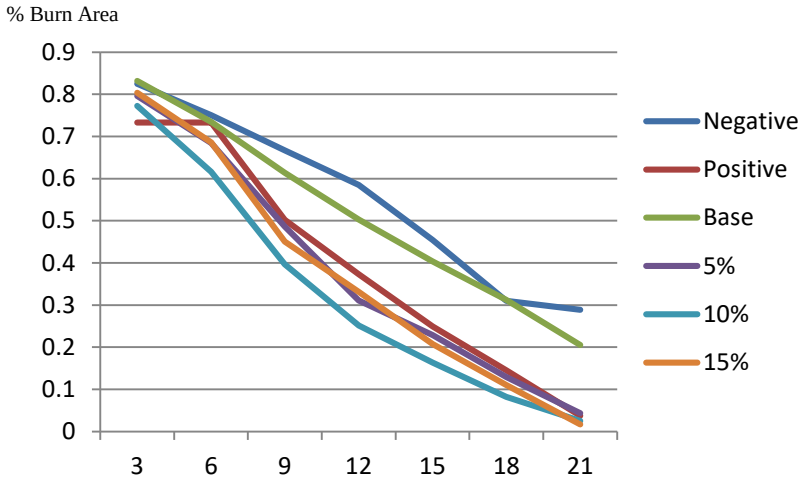

Figure 5. Graph of the percentage of burn area

Figure 5 illustrates the burn-healing process for each treatment from the 1st to the 21st day, showing reductions in the surface area of the burns. On the 21st day, the average percentage of burn healing for each treatment was calculated: the negative control, positive control, and membrane base had values of 71.12%±0.03, 96.23%±0.06, and 79.48%±0.01, respectively. Additionally, the percentage recovery values for the 5%, 10%, and 15% membranes were 95.62%±0.03, 97.44%±0.04, and 98.32%±0.03, respectively. These results indicate that the bioactive fraction of mangiferin from mango leaves has the potential to accelerate burn healing, consistent with several reports highlighting the potency of natural ingredient extracts in wound healing and therapy[30].

The healing effects of burns attributed to the bioactive fraction of mango leaves, including mangiferin, can be attributed to its anti-inflammatory, analgesic, and antibacterial properties. The anti-inflammatory activity is mediated by inhibiting COX-1, COX-2, and PGE-2 production, as well as inactivating the NLRP3 inflammasome [31,32]. The proliferation and maturation phases of burn healing are influenced by factors such as the type and extent of damage, the patient's overall health, and the tissue's regenerative capacity. The intervention of

the mangiferin bioactive fraction membrane plays a crucial role in initiating and facilitating this process. While all concentrations exhibited a healing effect, the 10% concentration proved to be the most potent, as evidenced by the significant reduction in burn size observed from the initial assessment until day 21[33,34].

3.6. ANOVA

The analysis revealed that the dataset consisted of normally distributed data, which included both homogeneous and non-homogeneous subsets. The assessment of normality using the Kolmogorov-Smirnov test indicated that the healing activity of the mangiferin bioactive fraction membrane followed a normal distribution, with a significance level of 0.2, which is greater than the threshold of 0.05. Additionally, the homogeneity test, as determined by the Levene statistical test, indicated that the data were homogeneous, with a significance level of 0.096, also greater than the 0.05 threshold.[35]

A two-way ANOVA comparing all test groups in terms of the percentage of burn wound healing demonstrated a significant effect within the treatment group. Additionally, Duncan's posthoc test indicated significant differences between most groups, except for the comparison between the 5% preparation and the positive control, as well as between the 10% and 15% preparations within the same subset. It was observed that burn healing improved consistently with each passing day[35].

Based on these results, the groups with preparations of 10% to 15% demonstrated the highest effectiveness and exhibited similar activity. However, they showed significant differences when compared to the 5% concentration preparation. Moreover, these higher concentrations offered several advantages compared to the comparator preparation35. Economically, a preparation with a 10% concentration would be more favorable.

4 CONCLUSIONS

Applying a bioactive fraction membrane with a mangiferin content of 208.31 µg/mL and concentrations of 5%, 10%, and 15% significantly affected the healing of superficial second-degree burns in rabbits, resulting in healing percentages of 95.62%±0.03, 97.44%±0.04, and 98.32%±0.03, respectively. Furthermore, a significant difference in healing time was observed among the treatment groups. Membranes with a concentration of 10% to 15% of the mangiferin bioactive fraction were the most effective, achieving percentages of 97.44%±0.03 and 98.32%±0.03 within 21 days.

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القياس الكمي للمانجيفيرين من كسر المنشط الحيوي من أوراق المانجو (منجيفيرا هندية ألـ.) وتقييم إمكانات التئام الجروح

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ملخص

تشير الحروق إلى الأضرار التي تلحق بسطح الجلد بسبب ارتفاع درجات الحرارة من الزيت والماء والكهرباء والنار والتعرض لأشعة الشمس والمواد الكيميائية. تتطلب علاجا سريعا ومناسبا لتجنب الآثار غير المرغوب فيها. لذلك، تهدف هذه الدراسة إلى تحديد كمية المانجيفيرين، التي يمكن أن تعالج الحروق، في كسر المنشط الحيوي من أوراق المانجو (منجيفيرا هندية ألـ.) وتقييم نشاطها في التئام الحروق. تشمل الطرق المستخدمة في قياس إستشراب الطبقة الرقيقة (تي ألـ سي – - TLC) مكثافية بصرية مع التحقق من ضبط الخطية ، وحدود الكشف والقياس الكمي (ألـ أو دي LoD و ألـ أو قيو LoQ) والدقة والتقدير الكمي. تمت صياغة كسر المنشط الحيوي في غشاء بتركيز 5 / 10 و 15%. تم تطبيق الغشاء على الأرانب التي سبق أن تعرضت لحروق 6 جروح وعولجت ضد 4 ذكور من الأرانب. تم قياس تقدم الشفاء بقطر الحروق باستخدام المسماك المورن في كل 3 أيام إلى 21 يوما. تم الكشف عن المركب النشط (المانجيفيرين) بطول موجة 257 نانومتر. أنتجت نتائج الاختبار المعادلة الخطية واي = 2935.7 + x76596 مع قيمة معامل الارتباط 0.9957 ، حد الكشف 2.01 ميكروغرام / مل µg/mL، قيمة حد التقدير 6.07 ميكروغرام / مل، معامل الاختلاف في المدى 0.59 – 3.33 %، ومدى دقة 99.18 – 100.9% مع مستويات المانجيفيرين 208.31 ميكروغرام / مل. تم اختباراستعدادات غشاء كسر المنشط الحيوي المانجيفيرين على حروق من الدرجة الثانية في الأرانب. أظهرت النتائج أن التراكيز 10% و 15% كانت الأكثر فعالية.

الكلمات الدالة: كسر المنشط الحيوي، المنجفيرين، الحروق، الغشاء، القياس الكمي.

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