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THE IMPACT OF TAMANU OIL *(Calophyllum inophyllum* L.) ON IMPROVING THE WOUND HEALING PROCESS IN DIABETIC MICE MODEL.

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Abstract

Diabetic ulcers pose a significant challenge in clinical practice due to impaired wound healing caused by hyperglycemia, infections, and oxidative stress. This study evaluates the effects of Tamanu oil (Calophyllum inophyllum L.) from Hue City on wound healing in a diabetic mouse model. Chemical analysis revealed that Tamanu oil contains bioactive compounds, including calophyllolid and hexadecanoic acid, with antioxidant, anti-inflammatory, and antibacterial properties. *In vitro* experiments demonstrated that Tamanu oil stimulates fibroblast proliferation and exhibits antibacterial activity. *In vivo* studies on diabetic mice showed faster wound closure and earlier hair regrowth compared to the control group, suggesting that Tamanu oil supports diabetic wound healing.

Key words: Tamanu oil, diabetic ulcers, wound healing, antioxidant, anti-inflammatory, antibacterial.

I. INTRODUCTION

Diabetic ulcers are among the most challenging types of injuries to study in clinical practice. Typically, the wound healing process involves four main stages: hemostasis, inflammation, proliferation, and remodeling [18]. Diabetes, however, disrupts most of these processes. Persistent hyperglycemia and associated diabetes complications, such as infection, inflammatory responses at the wound site, microvascular complications, and oxidative stress, severely impact patients' quality of life, aesthetics, and mental well-being [1]. This has driven extensive experimental research to discover new substances and develop novel treatment strategies, providing valuable insights into the molecular mechanisms of tissue damage and wound healing, as well as potential therapeutic targets. Animal studies currently play a crucial role in wound healing research, with mice remaining the most commonly used experimental model in wound healing research.

Tamanu Oil (*Calophyllum inophyllum*) has been shown to promote skin cell growth, stimulates collagen production, and aid in regeneration, anti-aging, infection prevention, and scar reduction. Research in Vietnam has shown the effectiveness of combining Tamanu oil with Cajuput oil for burn treatment in rabbit models [9]. Compounds derived from Tamanu trees, such as triterpenoids, steroids, flavonoids, coumarins, xanthones, fatty acids, esters, alkenes, and alicyclic compounds, provide health benefits including antiviral, anticancer, anti-inflammatory, antibacterial, antiplatelet, and antifungal effects. They are also used to treat conditions such as athletes foot, eczema, acne, psoriasis, burns, cracked skin, and dermatitis. Tamanu fruit contains approximately 75% oil, and recent studies indicate its analgesic properties for treating conditions like rheumatism, sciatica, conjunctivitis, burns, and preventing infant rashes [3], [15].

As diabetic wound healing is a complex process, this study aimed to explore the potential of Tamanu oil to promote tissue regeneration and accelerate wound closure in a mouse model of diabetes-induced impaired wound healing.

II. MATERIALS AND METHODS

Extract preparation

Ripe Tamanu fruits were collected and dried at 40-50^{0}C for 4 hours. Light-yellow seeds were selected and ground into small particles. The oil refining process was based on the study of Minh Nhat Nguyen et al [13] with modifications, including five main steps:

Deresination: Crude oil was treated with an azeotropic solvent mixture

(methanol) in varying ratios (1:1.5 to 1:4, w/v), stirred at 500 rpm, and heated to 40^0C for 5-15 minutes. Oil was separated using a separating funnel, and residual solvent removed via rotary evaporator.

Degumming: Distilled water (22 mL per 20 g oil) was added to remove hydratable phospholipids. The mixture was stirred at 50^0C for 15 minutes, centrifuged at 6000 rpm, and the degummed oil filtered under vacuum.

Neutralization: Free fatty acids were neutralized with 20% KOH solution. The mixture was stirred at 50°C for 15 minutes, then dehydrated under vacuum at 90^0C, and residual water adsorbed with sodium sulfate.

Bleaching: Neutralized oil was treated with activated carbon (1-5%, w/w) at 50^0C for 15 minutes to remove pigments and oxidation products. Bleaching efficiency was determined by spectrophotometric absorbance at 302 nm.

Deodorization: Bleached oil was heated to 249-254^0C under vacuum, steam distilled for 15 minutes, then cooled to 125-130^0C under low pressure.

Chemical analysis

The extract of Tamanu oil was tested by the Drug, cosmetic and Food Quality Control Center of Thua Thien Hue province (HueQC). The composition was analyzed by the Gas Chromatography-Mass Spectrometry method (GC-MS).

Determination of total flavonoid content (TFC)

The total flavonoid content in the oil was estimated by the aluminum chloride colorimetric method described by Marinova et al. (2005) with slight modifications [12].

Different concentrations of quercetin standards (20-200 μg/mL) and Tamanu oil extracts (1 mg/mL) were prepared in methanol. Next, 0.3 mL of 5% $NaNO_2$ was added, followed by 1 mL of the oil extract and 4 mL of distilled water. After five minutes, 0.3 mL of 20% $AlCl_3$ was added and kept for 6 minutes. Finally, 2 mL of 1M NaOH and distilled water (to 10 mL) were added. The optical absorbance of the resulting solution was measured at 510 nm after 10 minutes. Based on the measured absorbance values at each concentration, a QE calibration curve was constructed to determine the total phenolic content in the extracts. Results were expressed as milligrams of quercetin acid equivalents per gram of extract (mg QE/g extract). All tests were conducted in triplicate.

Determination of total polyphenol content (TPC)

A solution of 7.5% sodium carbonate was prepared using distilled water. The Folin-Ciocalteu reagent was also prepared by diluting it to a 1:10 ratio with distilled water. Gallic acid solutions (50-200 μg/mL) and Tamanu oil extracts (1 mg/mL) were prepared in methanol. For the experiment, 0.5 mL of the oil

extract solution was mixed with 2.5 mL of Folin-Ciocalteu reagent, followed by 1 mL of 7.5% Na_2CO_3. The mixture was incubated at room temperature in the dark for two hours. Absorbance was measured at 760 nm, and results were expressed as milligrams of gallic acid equivalents per gram of extract (mg GAE/g extract). All tests were conducted in triplicate.

DPPH radical scavenging assay

The antioxidant activity of Tamanu oil was determined through DPPH free radical scavenging according to Jahan et al (2010) with some modifications [7]. This method measures the reduction of DPPH in the presence of hydrogen-donating antioxidants. A 100 μM DPPH solution was prepared in methanol, and the extract was diluted to concentrations of 5-25 μg/mL. A mixture of 1.5 mL of 100 μM DPPH and 1.5 mL of extract was shaken, incubated in the dark at room temperature for 30 minutes, and the absorbance was measured at 517 nm. Ascorbic acid was used as a standard antioxidant. Radical scavenging activity was expressed as IC50, the concentration required to reduce DPPH by 50%. Lower absorbance indicated higher scavenging activity and the antioxidant activity percentage was calculated using the following formula:

$$\%\ of\ antioxidant\ activity\ = [(A0 - A)/A0] \times 100\%)$$

Where:

A_0 = the absorbance of blank sample solution without the compound to be tested;

A = the absorbance of the tested sample.

Anti-inflammatory activity

The anti-inflammatory activity was evaluated using a heat-induced albumin denaturation inhibition assay in an *in vitro* model, as modified from Tran Quoc Tuan et al. (2014) [18]. Diclofenac was used as the positive control in this study.

The composite consisted of 2 mL of 0.025M acetate buffer (pH 5.5), 1 mL of 0.16% bovine serum albumin (BSA) solution, and 1 mL of the sample at different concentrations (100, 50, 25, 12.5, and 6.25 μg/mL). Incubate the mixtures at 37^0C for 30 minutes. Heat the samples in a water bath at 67^0C for 3 minutes, followed by rapid cooling under running water. Measure the optical density (OD) at 660 nm. Construct a linear correlation equation (y = ax + b) between the percentage inhibition and sample concentrations. Determine IC_{50} value, where 50% of the protein is denatured. Each experiment was repeated three times for statistical accuracy.

$$\%\ \text{inhibition} = [(OD_{\text{blank}} - OD_{\text{sample}}) \times 100]/OD_{\text{blank}}$$

Where: OD_{blank} : optical density of the blank sample (buffer instead of extract)

OD_{sample}: optical density of the test sample

Antibacterial activity by the disc diffusion method

The bacterial strains were provided by Medical Testing Laboratory, Microbiology Department, Hue Central Hospital

In the disc diffusion assay, nutrient agar was prepared using peptone, meat extract, yeast extract, and agar. The medium and all equipment were sterilized before use. Antibacterial activity was evaluated at two concentrations (100% and 50%) against four bacterial strains:

Escherichia coli, Bacillus pumilus, Salmonella, and *Staphylococcus aureus.* Distilled water served as the negative control, while Ampicillin (10 mg/mL) was the positive control. Bacterial inocula were streaked onto the agar surface, and samples were applied. Plates were refrigerated for 6-8 hours and then incubated at 37^0C for 24 hours. Tests were conducted in triplicate, and antibacterial activity was expressed as the mean inhibition zone diameter (mm).

Cell culture and cell proliferative assay

The experiment was conducted following the method of Francois A. Auger (2008) with modifications tailored to laboratory conditions.

When the cell density reached 70-80%, fibroplast was subcultured into 6-well cell culture disk. Create an 800 μm-wide scratch by 200μL tip when the well density reached 70-80%. The wells were rinsed twice with PBS to clean the scratch area and remove debris. Two experimental groups of cells were observed to evaluate migration and proliferation within the scratched region, while the control group: DMEM F12 + 10% FBS + 1% ampicillin; and the experimental group: DMEM F12 + 10% FBS + 1% ampicillin + different concentration of Tamanu oil in each well.

Cell counts were performed automatically using the ImageJ software, enabling the evaluation of Tamanu oil's ability to stimulate cell proliferation at three different concentrations: 50 ppm, 100 ppm, and 150 ppm.

Experimental animals

Model Creation: 12 male Swiss mice weighing approximately 30 $\pm$ 2g (10 weeks old) were fed a high-fat diet for six weeks to induce hyperlipidemia. Mice are anesthetized using a mixture of Xylazil, Ketamil, and NaCl in a ratio of 2:3:3, following the method described by Phan Dieu Ngan (2019). Streptozotocin (STZ) was injected at low doses (35 mg/kg) to induce diabetes. Diabetes was confirmed if blood glucose levels exceeded 11 mmol/L [5].

Wound Induction: Full-thickness skin wounds (2 cm diameter) were created on the rats' backs. The mice were shaved on their backs, and sterile biopsy punches, forceps, and scissors were used to create standardized wounds. These tools were sterilized by immersion in boiling water at 100^0C for 20 seconds. A biopsy punch was used to create a hole, and the excised skin was removed with sterile forceps and scissors to create a uniform wound as 12 mice need to have comparable burn wounds. After the procedure, the mice were immediately

moved to a clean room maintained at 24-26^{0}C and were randomly divided into two groups: Control group: The wounds were healed naturally; Experimental group: Tamanu oil was applied topically to the wounds using a cotton swab, administered twice daily at 8:00 AM and 4:00 PM. Healing was monitored over 13 days using scoring, wound area measurement, and histological examination.

IV. RESULTS AND DISCUSSION

Chemical analysis The chemical analysis of Tamanu oil from Hue City identified key bioactive compounds, including Calophyllolid (80.98%), n-Hexadecanoic acid (4.32%), and trans-Cinnamic acid (6.11%), along with other organic acids and minor components like α-Cubebene.

Peak#	Name	R.Time	Area	Height	Area%
1	1,3,5,7-Cyclooctatetraene	5.526	56561232	27029612	5.73
2	Cyclohexene, 3,3,5-trimethyl-	6.005	3762333	1908948	0.38
3	Cyclopentane, bromo-	7.907	14715041	4313850	1.49
4	2-Cyclohexene-1-carboxaldehyde, 2,6,6-trimethyl	18.341	173938	58978	0.02
5	Copaene	18.494	170041	57888	0.02
6	.alpha.-Cubebene	19.601	990284	270338	0.10
7	trans-Cinnamic acid	20.716	60267321	3878564	6.11
8	Isoamyl cinnamate	25.026	1001089	333611	0.10
9	Allyl cinnamate	28.412	5423517	1597132	0.55
10	3,7-Nonadien-2-ol, 4,8-dimethyl-	30.039	2015330	674496	0.20
11	n-Hexadecanoic acid	31.939	42668303	5665388	4.32
12	Calophyllolid	35.685	799142744	54796002	80.98
			986891173		100.00

Table 1. Chemical compositions of Tamanu oils

Calophyllolid derived from Tamanu fruits in India has shown anti-inflammatory properties and promotes wound healing. Similarly, Tamanu oil from Hue City contains bioactive compounds with antibacterial and anti-inflammatory activities. Among these, hexadecanoic acid demonstrates significant biological properties, including antioxidant, cholesterol-lowering, antibacterial, and anti-inflammatory effects. It has been utilized in treating human HT-29 cancer cell lines and exhibits antibacterial activity against *Staphylococcus aureus, Escherichia coli,* and *Enterococcus faecalis in vitro* [6]. Cinnamic acid and its bioactive metabolites are commonly found in human diets, such as in vegetables, fruits, honey, and whole grains. Global studies have highlighted the pharmacological potential of cinnamic acid and its derivatives, which include antibacterial, antioxidant, anticancer, anti-atherosclerotic, antitubercular, and antifungal activities [17].

Quantification of total phenolic and flavonoid content

The total phenolic content (TPC) was quantified using gallic acid (GA) as a standard, with concentrations ranging (0, 50, 100, 150, 200 μg/mL), producing

a linear regression equation of y = 0.009x + 0.1222. The TPC of Tamanu oil was determined to be 102.40 ± 1.12 mg GAE/g.

The total flavonoid content (TFC) was similarly quantified using quercetin as a standard, with concentrations ranging (0, 50, 100, 150, 200 μg/mL), producing a regression equation of y = 0.0051x + 0.0243. The TFC of the Tamanu oil was calculated to be 105.76 ± 0.72 mg QE/g.

Natural antioxidants are plant-derived bioactive compounds that help inhibit the progression of cancer, diabetes, and other chronic diseases. Comparison with global research, such as the study by Ruangsuriya J et al. (2023), shows similar results, with reported TPC and TFC values of 109.16 ± 1.21 mg GAE/g and 96.88 ± 0.89 mg QE/g, respectively. Various types of phenolic compounds derived from plants, including phenolic acids, coumarins, flavonoids, xanthones and polyphenols, have been shown to prevent various degenerative diseases and exhibit strong antioxidant activity, preventing chronic conditions, including cancer, diabetes, and cardiovascular diseases [16]. These findings confirm that Tamanu oil from Hue City is rich in bioactive compounds, making it a valuable source of natural antioxidants.

In vitro **antioxidant activity of Tamanu oil.**

The free radical scavenging activity of Tamanu oil was evaluated using the IC_{50} value, which represents the concentration required to inhibit 50% of free radicals. Based on the linear regression equation y = 0.5864x + 20.505, the IC_{50}value was calculated as 50.08 μg/mL. These results indicate that Tamanu oil exhibits strong antioxidant potential. The findings align well with global research, confirming its efficacy as a natural antioxidant source. This supports its application in preventing oxidative stress-related conditions, such as aging, inflammation, and chronic diseases [3], [14].

Anti-inflammatory activity

Tamanu oil *(Calophyllum inophyllum)* demonstrated strong anti-inflammatory activity in reducing edema. Its efficacy was evaluated using an *in vitro* albumin denaturation inhibition assay, as adapted from Tran Quoc Tuan (2014), with diclofenac as the positive control.

The result showed that the protein denaturation inhibition efficiency of Tamanu oil increased proportionally with its dilution. Based on the regression equations y = 0.2664x + 41.857 and y = 0.317x + 19.485, the IC_{50} values for diclofenac and Tamanu oil were determined to be 31.58 μg/mL and 96.26 μg/mL, respectively. The IC_{50} result of Tamanu oil was higher than that of diclofenac, indicating relatively lower anti-inflammatory activity. Inflammation, a key factor in the progression of chronic conditions such as arthritis, gastritis, and hepatitis, could potentially be mitigated through the use of Tamanu oil as a natural therapeutic agent.

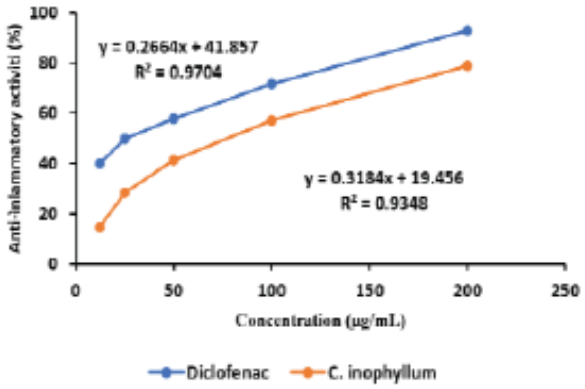

Figure 1. Anti-inflammatory activity of Tamanu oil.

Antibacterial activity

The results showed that Tamanu oil from Hue City exhibited strong antibacterial activity against both Gram-positive and Gram-negative bacteria, particularly against the key pathogens commonly found in diabetic ulcers: *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Escherichia coli.* These findings highlight the potential of Tamanu oil as a natural agent for combating diabetic ulcer infections caused by these bacteria.

The study revealed that Tamanu oil effectively inhibited all tested bacteria, with the strongest activity against *Escherichia coli, Bacillus pumilus, Salmonella,* and especially *Staphylococcus aureus.* This pathogen is a leading cause of bloodstream infections, endocarditis, and various skin and soft tissue infections. It breaches the skin barrier, causing conditions like ulcers, blistering, toxic shock syndrome, and exfoliative dermatitis. According to Livimbi and Komolafe (2007), *Staphylococcus aureus* is the most commonly isolated bacterium from wounds, followed by *Pseudomonas aeruginosa, Proteus mirabilis, Streptococcus spp., E. coli, Salmonella,* and *Klebsiella spp* [11]. Similarly, *Pseudomonas aeruginosa* acts as an opportunistic pathogen, causing infections in skin and soft tissues at wound sites [2]. These findings align with global research, which highlights the antibacterial properties of hexadecanoic acid, a key compound in Tamanu oil, against *Staphylococcus aureus, Escherichia coli,* and *Enterococcus faecalis* in *in vitro* studies.

Fibroblast scratch assay proliferation result.

A scratch assay was conducted on a monolayer of cultured cells to evalu-

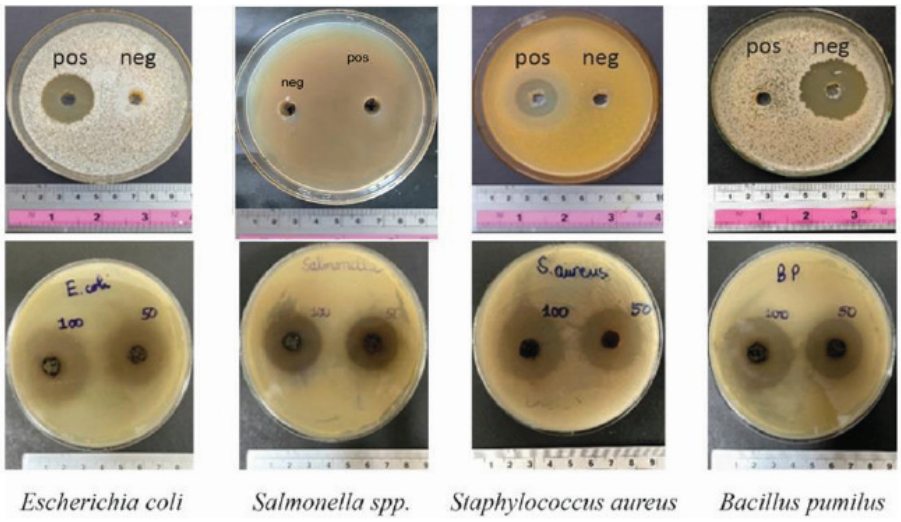

Figure 2. Antibacterial activity of Tamanu oil on different bacteria strains.

ate the wound-healing potential of Tamanu oil. After 48 hours of cell culture, Tamanu oil was applied, and observations were made over the next 48 hours. Cell migration into the wounded area was monitored using an inverted microscope. After 36 hours, the control group showed no significant change in wound size. However, in the groups treated with Tamanu oil at concentrations of 50 ppm, 100 ppm, and 150 ppm, rapid cell division and migration were observed. Significant wound size reduction was seen as early as 6, 9 and 12 hours, with further narrowing at later intervals. Representative images demonstrate the effectiveness of Tamanu oil in promoting cellular migration and wound closure.

After four days of culture with Tamanu oil supplementation, all experimental groups showed a significant increase in cell numbers compared to day 0. On 50 ppm, 100 ppm and 150 ppm concentration, the average cell count increased to 148,473 $\pm$ 4,886 cells/cm^2, 217,547 $\pm$ 3,417 cells/cm^2, 127,436 $\pm$ 10,743 cells/cm^2, respectively, while the average cell count of control group was 110,700 $\pm$ 10,465 cells/cm^2. The results indicate that Tamanu oil significantly promotes cell proliferation, as demonstrated by the marked difference in cell numbers between the treated and control groups. This finding highlights the essential role of natural compounds in regulating cell proliferation, emphasizing their importance as physiological and pharmacological modulators.

Wound healing results in rat models

Streptozotocin (STZ) is a chemical agent widely utilized in biomedical research for to selectively destroy pancreatic beta cells, impairing insulin pro-

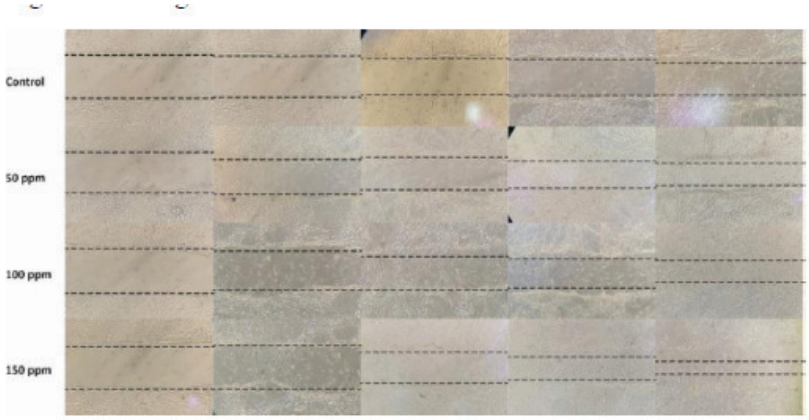

Figure 3. Comparison of wound after gap creation between Control group and different Tamanu oil concentration.

duction and inducing type 1 diabetes mellitus (T1DM) in animal models [10]. It is a widely used method for studying T1DM due to its reliability and reproducibility. STZ induces diabetes through two primary mechanisms: direct necrosis of beta cells and immune-mediated pathways [5].

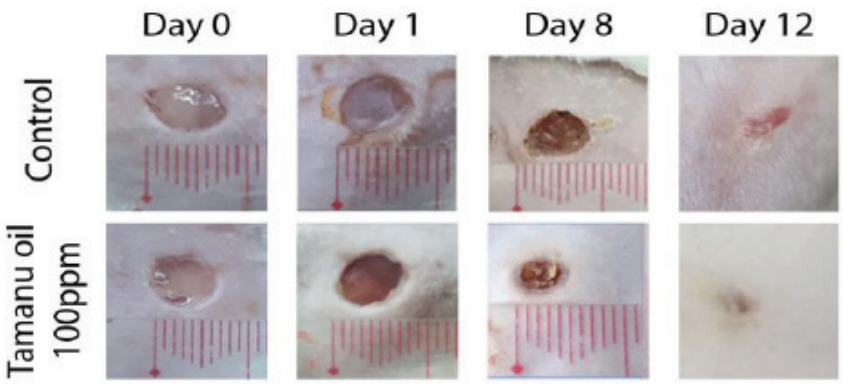

Figure 4. The enhancement of wound closure by Tamanu oil

The administration of STZ in experimental models leads to insulin deficiency and persistent hyperglycemia, mimicking the pathophysiological features of type 1 diabetes. This model is essential for investigating the mechanisms of T1DM and evaluating potential therapeutic interventions. The severity of

the wound was assessed based on both their extent and depth. The extent was estimated by the total body surface area affected, while the depth was determined by the degree of damage to the epidermis or dermis layers. Recognizing ethical and social concerns in animal studies, measures were taken to minimize animal usage and harm by refining experimental protocols [8]. The objective was to establish a thermal injury model and evaluate the efficacy of Tamanu oil in promoting wound healing in diabetic ulcers.

The wounds were monitored for full healing over a 12-day follow-up period after ulcer creation, with the evaluation based on the reduction in burn wound diameter. Tamanu oil demonstrated positive therapeutic effects, significantly aiding the healing process with no scarring observed. The results suggest that Tamanu oil-based products contain compounds with strong anti-inflammatory, antioxidant, and antibacterial properties, crucial for preventing skin infections during wound healing. Calophyllolid, a key compound in Tamanu oil, accelerates wound healing, reduces both internal and external inflammation, and helps the body target and eliminate pathogenic microorganisms, thus supporting burn wound recovery [4].

CONCLUSION

Tamanu oil significantly enhances wound healing in diabetic models through its antioxidant, anti-inflammatory, and antibacterial properties. Its bioactive compounds, such as Calophyllolid and hexadecanoic acid, contribute to tissue regeneration and infection prevention, suggesting its potential as a natural therapeutic agent for diabetic ulcers.

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