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Evaluation of the combination patch of betel leaf extract (*Piper* sp.) - ultrasonic assisted extraction as a transdermal delivery system on fibroblast cell formation and collagen density

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ABSTRACT: The combination of red betel (*Piper crocatum*) and green betel (*Piper betle* L.) leaves is believed to act synergistically in enhancing wound healing. Transdermal patches offer advantages over conventional topical forms due to their sustained drug release, ease of application, and prolonged therapeutic effect. This study evaluated the histopathological effects of a combination patch containing betel leaf extracts for incision wound treatment in Wistar rats. The studies used an true experimental post-test-only control group design, five groups were tested: a blank patch (K−), a povidone-iodine patch (K+), and combination extract patches at concentrations of 7.5% (K1), 15% (K2), and 30% (K3), applied over 14 days with six animals per group. Patch characteristics were assessed through organoleptic tests, thickness, weight uniformity, folding endurance, and pH, while wound healing was evaluated through histological analysis of fibroblast cell counts and collagen density. Statistical analysis was conducted using the Kruskal-Wallis test followed by Mann-Whitney post-hoc tests. The 30% combination patch (K3) showed the most significant healing effect, with fibroblast counts exceeding 50 cells and dense collagen formation scoring +3. These results indicate that a transdermal patch combining *Piper crocatum* and *Piper betle* L. extracts effectively promotes wound healing by enhancing fibroblast proliferation and collagen synthesis.

KEYWORDS: Collagen; fibroblast; patch; *Piper* sp.; wound healing.

INTRODUCTION

Wounds are physical damage resulting from the rupture or disruption of the skin, in which the intact epithelial cells are damaged, and the balance of organs and skin function is disrupted. An instance of an open wound is a laceration or incision caused by a break in tissue continuity due to surgery [1]. According to the 2023 Health Statistics Profile published by BPS-Statistics Indonesia on December 20, 2023, the prevalence of injuries in Indonesia in 2023 was 7.8%. It shows a slight increase compared to previous data in 2018, in which the prevalence of injuries was 8.2% [2].

The wound healing process is a sophisticated biological system that constantly repairs injured tissue via many phases, including homeostasis, inflammation, proliferation, and remodeling. Treatment of cuts can be done by applying topically in creams, ointments, gels, and transdermal patches. Transdermal patches can distribute drugs through the skin slowly over a while, and then the drug will be absorbed by the skin and into the bloodstream [3]. Drug administration through transdermal patch preparations has several advantages in its ability to control the release of drugs in a controlled manner, avoid side effects that may occur in the digestive tract, and provide the convenience of more extended use [4].

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Medicinal plants have fewer adverse effects in comparison to synthetic pharmaceuticals. Thus, many people switch to natural ingredients in treating wounds. Betel leaf (Piper sp.) is used to treat wounds. Betel leaf contains saponins, alkaloids, tannins, and flavonoids. The concentration of tannin and saponin compounds stimulates collagen formation, which plays a role in wound healing. Flavonoid compounds help enhance wound healing by the proliferation of fibroblast cells. Meanwhile, the content of tannin and saponin compounds spurs collagen formation, which plays a role in wound healing. The use of a combination of betel leaves, both red betel and green betel, in healing the incision wound is believed to work synergistically to encourage optimal wound healing [5], [6]. Previous studies have shown that red betel (*Piper crocatum*) extract can accelerate wound healing by increasing collagen formation and achieving up to 98% wound closure. This effect is associated with the upregulation of healing-related proteins and the reduction of proteins that inhibit recovery [7]. Similarly, green betel (*Piper betel* L.) extract has been proven effective in promoting healing, especially in diabetic wound models, by increasing beneficial compounds and reducing tissue-damaging substances [8].

Although red betel (*Piper crocatum*) and green betel (*Piper betel* L.) have demonstrated individual effectiveness in promoting wound healing, their combined use in a single transdermal patch formulation has not yet been explored. This indicates a lack of prior investigation into integrating these herbal extracts for wound care. Combining these two extracts may provide enhanced therapeutic outcomes by utilizing their complementary mechanisms of action. Accordingly, this study aims to assess the histological effects of a transdermal patch containing both extracts, focusing on in vivo evaluation of fibroblast proliferation and collagen density.

▪ MATERIALS AND METHODS

Materials

The tools that will be used in this study are a microscope (Olympus CX41), an Optilab camera (Optilab Pro, Phoenix, Arizona), a digital ultrasonic cleaner (Skymen, China), an analytical balance (Mettler Toledo, Germany), and an autoclave (JIBIMED, Jiangyin City, Jiangsu Province, China). This research used red betel leaves and green betel leaves sourced from Jekan Raya Village in Palangka Raya City, Central Kalimantan, and have gone through a plant determination process at the InaCC BRIN Characterization Laboratory (Cibinong, Bogor-West Java), male white rats (*Rattus norvegicus*) Wistar strain aged 2 - 3 months, weighing between 200 - 250 grams (n = 6 rats/group) which are categorized into five groups, namely negative control group, positive control, treatment group with a concentration of 7.5%; 15%; and 30%. Some materials for making patches include hydroxypropyl-methylcellulose (HPMC) (Merck, Germany), polyvinylpyrrolidone (PVP) (Sigma-Aldrich, Singapore), propylene glycol (Merck, Germany), dimethyl sulfoxide (DMSO) (Merck, Germany), and ethanol 96% (Brataco, Indonesia).

Ultrasonic Assisted Extraction (UAE)

The extraction process was accomplished using the ultrasonic-assisted extraction (UAE) method on each betel leaf powder with a sample/ethanol 96% solvent ratio of 1 g/4 mL, an extraction time of 20 minutes, and a temperature of 40°C with a frequency of 47 KHz. After obtaining the liquid extraction results, the evaporation continued using a rotating vacuum evaporator at 60 revolutions per minute at a temperature of 60°C. Then, for each betel leaf extract, both red betel and green betel are mixed (1:1) [9].

The quantitative phytochemical

Alkaloid test

Alkaloid content testing was done by putting 10 grams of the sample. Subsequently, 200 mL of a 10% acetic acid solution was introduced into a 250 mL beaker. The beaker was sealed and allowed to stand for 4 hours before filtration. A portion of the resulting extract was evaporated using a water bath; then ammonium hydroxide was added to produce a precipitate. The residue was washed using dilute ammonium hydroxide and filtered. The remaining residue was then evaporated to a fixed weight [10].

Flavonoid test

Flavonoid content testing was done by taking a prepared sample of 500 µL using a micropipette, pouring it into a test tube, and adding 2 mL of distilled water. After that, 150 µL of 5% $NaNO_2$ solution was added and left for 6 minutes. Subsequently, allow 150 µL of 10% $AlCl_3$ solution to stand for 6 minutes. Subsequently, 2 ml of 4% NaOH should be included and diluted with distilled water until the tube's total volume attains 15 ml. The mixture was allowed to stand for 5 minutes, after which the absorbance was assessed using a UV-Vis spectrophotometer at a wavelength of 520 nm [10] .

Saponin test

The test for saponin content was done by adding 10 grams of sample to a 250 ml beaker. Subsequently, 200 cc of 20% ethanol was included. The sample was evaporated in a water bath at 55°C for 4 hours. Afterward, the mixture solution was filtered, and the residue was subjected to re-extraction. The extract obtained was evaporated again with a water bath at 90°C until the volume decreased to 40 ml. The concentrate was then put into a separatory funnel to extract the water layer. Next, 60 ml of n-n-butanol was included and blended with the extract. This solution was washed with 10 ml of 5% NaCl before evaporating. Finally, the sample was dried in the oven until it reached a stable weight [10].

Tannin test

Tannin content testing was done by adding 1 ml of extract solution into a container, then continuing with 0.4 ml of Folin-Ciocalteu reagent and leaving it for 4-8 minutes. After that, 4 ml of 7% sodium carbonate (Na_2CO_3) solution was incorporated until well blended. Subsequently, 10 ml of distilled water was included and allowed to stand for 2 hours at ambient temperature. The solution's absorbance was quantified with a UV-Vis spectrophotometer at a wavelength of 744.8 nm [10].

The formulation of patch

In previous studies, a 15% red betel concentration effectively healed perineal wounds in female white rats (*Rattus norvegicus*) Wistar Strain [11]. Other research proves that green betel leaves with a concentration of 15% are the most effective for wound healing [12]. Based on the two studies, dosage modifications are made in this study, using multilevel doses of 7.5%, 15%, and 30% with a ratio of 1:1 for red betel and green betel. Table 1 shows the formulations for several treatment groups used in this study.

Table 1. Patch preparation formulation

Composition	F0 (Without extract)	F1 (7.5%)	F2 (15%)	F3 (30%)	Description
Combination extract *Piper* sp.	-	7.5 g	15 g	30 g	Active substance
HPMC	0.3 mg	0.3 mg	0.3 mg	0.3 mg	Polymer
PVP	0.1 mg	0.1 mg	0.1 mg	0.1 mg	Polymer
Propylene Glycol	0.5 mL	0.5 mL	0.5 mL	0.5 mL	Plasticizers, preservatives
DMSO	0.1 mL	0.1 mL	0.1 mL	0.1 mL	Penetration enhancer
Ethanol 96%	Ad 100 mL	Ad 100 mL	Ad 100 mL	Ad 100 mL	Solvent

The preparation of a patch

The stages of preparing a combination patch of betel extract at a concentration of 7.5% were performed by grinding PVP until it was smooth. Then, HPMC was added and continuously crushed until homogeneous and smooth. Then, 1 mL of distilled water was included and completely pulverized until uniform, forming a gel. Add 7.5 grams of red betel leaf and green betel leaf extract, stir until homogeneous, add propylene glycol until homogeneous, and add DMSO, and stir until homogeneous. After that, 96% ethanol was added to 100 mL and then poured into a mold as a petri dish. The bottom, wrapped with aluminum foil, was left for about one hour until no bubbles remained, and then dried at room temperature for approximately 48 hours until completely dry. After the patch's application is considered dry, please slowly remove it from the Petri dish's mold and cut it into 3 x 1 cm^2 (P x L). Subsequently, the patch preparation was attached to a hypafix patch with a length of 5 x 2 cm^2 (P x L). This patch preparation step can be repeated for 15% and 30% concentrations [13].

The evaluation of patch test

This evaluation is carried out to determine the characteristics and physical properties of the transdermal preparation patch combination of red and green betel leaves, with several formulations carried out, and to assess the preparation's quality. Evaluation of the physical properties of the transdermal patch consists of organoleptic tests, thickness tests, weight uniformity tests, pH tests, and folding resistance tests. The results of this test will be used to determine the suitability of the preparation. Several stages in the patch preparation evaluation test are as follows [14].

Organoleptic test

The patch preparation was observed visually using a magnifying glass. Organoleptic tests include color, aroma, and surface condition.

Weight uniformity test

The patch's weight was measured using an analytical balance, weighing each batch of each formula. Then, the average weight of the patch was calculated. Weighing was done three times in replication. Good weight uniformity has a coefficient variation (CV) value of <5%. This CV value obtained from the patch weight uniformity test meets the requirements.

Thickness test

The thickness of the patch is measured using a caliper tool. It is measuring the thickness of each patch at five different points. The standard thickness of a good patch is <1mm.

pH test

Place a universal pH on the surface of the expanded patch matrix for 1 minute; then, the pH is recorded. The pH of a good patch is 4-6.

Fold resistance test

The patch is folded repeatedly in the same place until it breaks. The durability of a good patch that meets the standard is the value >300 folds.

Haematoxylin-Eosin (HE) staining

Skin tissue samples were subjected to hematoxylin staining for 5 minutes, washed with water for 10 minutes, and then stained with eosin for 2 minutes. Samples were dehydrated using graded alcohol, cleared with xylol, and covered with adhesive cover glass. Then, observations were made on fibroblast cells calculated with a scoring system (Table 2) [15].

Table 2. Scoring cell fibroblasts.

Score	Description
0	No, there is a cell
1	5-10 cell
2	10-50 ell
3	>50 cell

Masson's trichrome staining

Skin tissue samples were fixed with 10% BNF, deparaffinized with distilled water, and then subjected to multiple stains, including Weigert's iron hematoxylin, Biebrich scarlet-acid fuchsin, aniline blue, and glacial acetic acid. After dehydration with alcohol and xylol, mounting was performed. Then, observations were made on collagen density calculated with a scoring system (Table 3) [15].

Table 3. Scoring the density of collagen.

Score	Information
+0	No collagen fibers were found in the wound area
+1	The density of collagen fibers in the wound area is low (less than 10% per field of view)
+2	Collagen fiber density in the wound area is moderate (10-50% per field of view)
+3	Collagen fiber density in the wound area is dense (50-90% per field of view)
+4	Collagen fiber density in the wound area is very dense (90-100% per field of view)

Data analysis

Statistical data analysis used in this study was SPSS 26 computer software for Windows. The statistical results of the normality and homogeneous tests showed that the data obtained were not regular and homogeneous, so the Kruskal-Wallis and Mann-Whitney post-hoc tests were conducted [16].

▪ RESULTS

The quantitative phytochemical results

The quantitative phytochemical tests of red and green betel leaf extracts contain alkaloids, flavonoids, saponins, tannins, and triterpenoid compounds. The results are presented in Table 4.

Table 4. The results of quantitative phytochemical.

Phytochemicals compound analysis	Level compound	
	Extract leaf red betel	Extract leaf green betel
Alkaloid (%)	38.733±0.732	49.153±0.484
Flavonoid (mg/ml QE)	98.083±0.381	129.250±0.5
Saponins (%)	31.353±0.671	35.743±0.427
Tannin (mg/ml GAE)	0.071±0.005	0.248±0.008
Triterpenoid (mg/ml)	963.133±1.154	967.800±1.732

The patch evaluation test results

The results of the patch preparation evaluation test are presented in Table 5.

Table 5. The results of the patch test.

Patch preparation evaluation	F0	F1	F2	F3	Description
Organoleptic test					
Color	Clear/not colored	Yellow clear	Yellow	Yellow brownish	Qualify
Aroma	No smelly	Smells spicy, typical betel	Smells spicy, typical betel	Smells spicy, typical betel	Qualify
Form	Fine, elastic, and springy	Fine, elastic, and springy	Fine, elastic, and springy	Fine, elastic, and springy	Qualify
Thickness Test (mm)	0.23	0.35	0.50	0.52	Qualify
Weight uniformity test (g)	0.33	0.50	0.49	0.54	Qualify
Folding resistance test	324	335	342	354	Qualify
pH Test	5.0	5.1	5.3	6.4	Qualify

The histopathological of collagen density with Masson's Trichome (MT) stain

Based on the study result, collagen density was observed across six fields of view under 400x magnification. Straight elongated fibers characterize the collagen, but the color is faint, slightly wavy, white, and homogeneous. Figure 1 shows the microscopic picture of collagen density with Masson's Trichome staining with 400x magnification in several treatment groups.

Based on microscopic observations, the collagen density that has been analyzed is illustrated (Figure 1). In the K-group (patch formulation without active ingredients), the collagen fiber density in the wound area was categorized as low (<10% per microscopic field), receiving a score of +1 compared to the other treatment groups. In contrast, the K+ group (povidone-iodine patch), K1 group (patch containing 7.5% betel leaf extract), and K2 group (patch containing 15% betel leaf extract) demonstrated moderate collagen fiber density (10–50% per microscopic field), with a score of +2. The K3 group (patch containing 30% betel leaf extract) exhibited the highest collagen fiber density among all treatment groups, classified as dense (50–90% per microscopic field), with a score of +3.

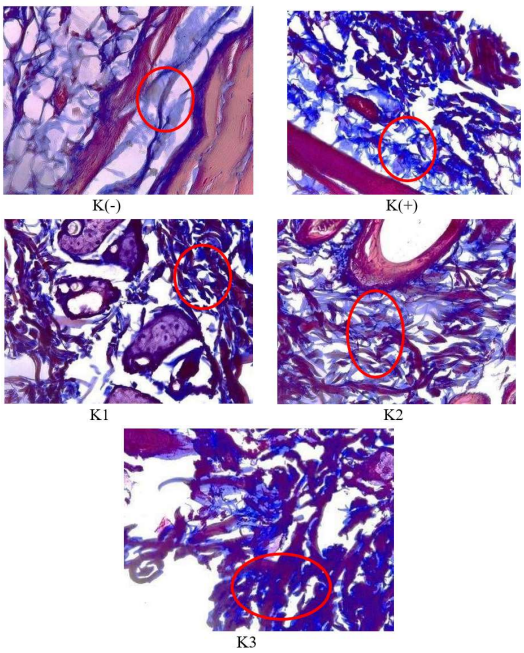

Figure 1. Histopathological Findings of Collagen Density Visualization with Masson's Trichrome (MT) Staining at 400x Magnification for Patch Formulations at Various Concentrations, Positive Control, and Negative Control (Red Circles = Collagen Density; K(-) = Patch without Betel Leaf Extract Combination; K(+) = Povidone iodine Patch; K1 = Patch with 7.5% Betel Leaf Extract Combination; K2 = Patch with 15% Betel Leaf Extract Combination; K3 = Patch with 30% Betel Leaf Extract Combination)

Analysis of collagen density parameters

The results of the statistical analysis based on the average collagen density are presented in Table 6.

Table 6. Analysis results of average collagen density.

Treatment groups	Average collagen density (%) (Mean±SD)
K(-)	8.33±2.877
K(+)	20.00±10.000
K1	30.00±10.000
K2	43.00±35.595
K3	78.33±20.207[a]

Description :
K(-) = Patch without Betel Leaf Extract Combination
K(+) = Povidone iodine Patch
K1 = Patch with 7.5% Betel Leaf Extract Combination
K2 = Patch with 15% Betel Leaf Extract Combination
K3 = Patch with 30% Betel Leaf Extract Combination
[a] = Significantly different from the negative control (-)

The histopathology of fibroblast cells with Haematoxylin-Eosin (HE) staining

Based on the observation of the slides stained with Haematoxylin-Eosin (HE) across six fields of view, the fibroblast cells were observed to have a large, flat, and branching shape. It appeared spindle-shaped when viewed from the side. The cell nuclei were elongated, with one or two nucleoli present. The observations were conducted for each experimental group, as illustrated in Figure 2.

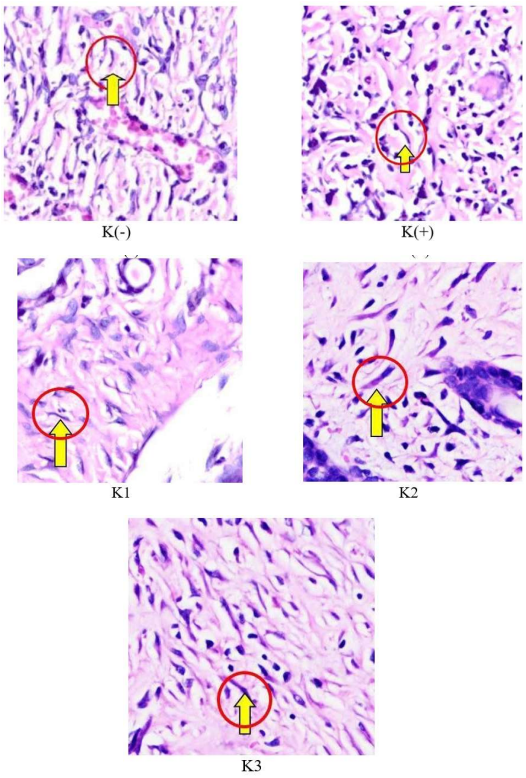

Figure 2. Histopathological results of collagen density with hematoxylin-eosin staining for patch formulations of various concentrations (red circles = fibroblast; K(-) = patch without betel leaf extract combination; K(+) = povidone iodine patch; K= patch with betel leaf extract combination (K1 = 7.5%; K2 = 15%; K3 = 30%)

Figure 2 illustrates the microscopic findings of fibroblast cells. In the K-group (patch formulation without active ingredients) and the K+ group (povidone-iodine patch), approximately 5–10 fibroblast cells were observed under 400x magnification. It can be concluded that the scoring for both K- and K+ is +1. Subsequently, the microscopic findings for the K1 group (patch with a 7.5% combination of betel leaf extract) and the K2 group (patch with a 15% combination of betel leaf extract) revealed the presence of approximately 10–50 fibroblast cells under 400x magnification. Therefore, the scoring for both K1 and K2 was determined to be +2. Following this, the microscopic findings for the K3 group (patch with a 30% combination of betel leaf extract) showed more than 50 fibroblast cells, resulting in a score of +3. Fibroblast cells had large dimensions, flattened structures, and spindle-like projections when observed laterally. The fibroblast nuclei were also elongated in shape and contained one or two nucleoli.

Analysis of fibroblast cell parameters

The statistical analysis results based on the average fibroblast cell are presented in Table 7.

Table 7. Analysis results of the average fibroblast cell.

Treatment groups	Average fibroblast cell (%) (Mean±SD)
K(-)	4.33±0.577
K(+)	10.00±1.732
K1	12.67±7.234
K2	16.00±6.245
K3	94.33±6.658[a]

Description :
K(-) = Patch without Betel Leaf Extract Combination
K(+) = Povidone iodine Patch
K1 = Patch with 7.5% Betel Leaf Extract Combination
K2 = Patch with 15% Betel Leaf Extract Combination
K3 = Patch with 30% Betel Leaf Extract Combination
[a] = Significantly different from the negative control (-)

DISCUSSION

This study continued research where green betel leaf extract yield using ultrasonic-assisted extraction (UAE) was 3.73%, and red betel leaf was 3.05% [9]. In another study, the yield of betel leaf extract using the maceration method was 2.44%. This indicated that the extraction yield using the non-conventional extraction technique in the UAE was higher than that obtained through conventional methods. Several factors influence the extraction yield, including the solvent type, the solvent-to-sample ratio, extraction time and temperature, and the particle size of the material. The UAE method worked by utilizing ultrasonic waves, which enhance the extraction of active compounds. Additionally, the increased extract yield obtained with UAE was influenced by the frequency and intensity of the ultrasonic waves applied [17].

This study showed that the concentration of secondary metabolites in green betel leaf extract was higher than in red betel leaf extract. Specifically, the alkaloid content in red betel leaf extract (38.733%) was lower than in green betel leaf extract (49.153%). The flavonoid content in green betel leaf extract (129.250 mg/ml QE) was also higher than in red betel leaf extract (98.083 mg/ml QE). Furthermore, the saponin content in red betel leaf extract (31.353%) was lower than in green betel leaf extract (35.743%). The tannin content in green betel leaf extract (0.248 mg/ml GAE) was significantly higher than in red betel leaf extract (0.071 mg/ml GAE). Additionally, the triterpenoid content in green betel leaf extract (967.800 mg/ml) was slightly higher than in red betel leaf extract (963.133 mg/ml). The flavonoids were known for their antioxidant properties; green betel leaves are commonly used in traditional medicine as a natural antioxidant [18].

In evaluating the patch formulation, several key parameters, such as organoleptic properties, patch thickness, weight uniformity, folding resistance, and pH, were assessed to ensure the quality and stability of the product. The organoleptic test showed that as the concentration of betel leaf extract increased, the patches' colour and odour became more intense, indicating a more potent extract [19],[20]. The patch thickness for all formulations was below 1 mm, within the acceptable range for effective use [21]. The weight uniformity test showed consistent results, with a coefficient of variation (CV) <5%, ensuring accurate and uniform dosage delivery [22]. Folding resistance tests demonstrated that the patches maintained their integrity and could withstand over 200 folds [23]. The pH of the patches ranged from 5.0 to 6.4, aligning with the natural pH of human skin, ensuring safety and minimizing the risk of irritation [24]. Overall, the evaluation confirms that the patch formulations meet quality standards for transdermal use, with betel leaf extract as the active ingredient, supporting its potential for clinical application.

A transdermal drug delivery system is an alternative for administering drugs through the skin layer. The active substance of this drug is carried through the skin into the bloodstream. Then, it enters the body's systemic circulation before reaching the target location through diffusion. Transdermal has several advantages compared to other giving routes, including the ability to use a sustained dose of drugs for a long time, being non-invasive, not going through the first-pass metabolic process, preventing damage to drugs that are not resistant to the pH of the digestive tract, and preventing irritation in the digestive tract. The transdermal patch can contain high doses of active substances that remain on the skin for a long time. Transdermal patch preparations have advantages in providing a comfortable and effective way of delivering medicine in various diseases. However, the development of this preparation in the future will face several challenges, such as the possibility of toxicity caused by inappropriate doses, low drug penetration, the emergence of skin irritation, poor adhesion, and treatment failure. Therefore, sustainable research is needed to optimize the safety and efficacy of the patch drug delivery system with transdermal routes. However, this has several challenges and weaknesses, such as limited doses. Over time, the discovery of new advances and innovations in the development of transdermal delivery systems has led to the ability to control drug release, higher loading, and increased drug penetration. Various research innovations, such as smart patches, have been carried out using technology to develop transdermal patch preparations for treating wounds. A traditional transdermal patch aims to store and release drugs. Wound healing is a complex and dynamic regenerative process influenced by some physical and chemical parameters. Smart patches are technological innovations equipped with sensors to monitor the patient's condition, especially for patients who lie in bed and require the appropriate drug delivery [25]. Previous research reported several advantages of smart patches, such as being cheap, flexible, and able to be printed on the skin to measure changes in wound pH and fluid volume. Flexible sensors can easily be inserted into the wound pads [26].

In incision wound healing, the two crucial factors were collagen density and the increase in fibroblast cells. Collagen density plays an essential role in the wound-healing process. Collagen is the main structural protein in the body, forming the framework of connective tissues, including the skin. During wound healing, the production and remodeling of collagen are essential for repairing and regenerating damaged tissue. Collagen was responsible for providing strength and structure to the healing tissue. During the inflammatory and proliferative phases of wound healing, fibroblasts began synthesizing collagen to form new granulation tissue. The higher the collagen density in the granulation tissue, the stronger and more resilient the wound became to external stresses [27]. Furthermore, collagen density plays a crucial role in scar tissue formation. As the wound healing process progresses into the remodeling phase, the collagen synthesized by fibroblasts undergoes reorganization into scar tissue. The proper amount and distribution of collagen were essential for determining the elasticity and strength of the scar tissue. The precise quantity and distribution of collagen significantly influenced the elasticity and strength of scar tissue. Insufficient or excessive collagen density can lead to the formation of scar tissue that does not adequately replicate the function of the original tissue [28].

The increase in fibroblast cells was significantly related to the wound-healing process. Fibroblasts were essential cells involved in the formation of connective tissue and the healing of wounds. During the inflammatory phase, the wound undergoes an immune response involving the release of cytokines and growth factors that stimulate the proliferation of fibroblasts from the surrounding tissue. This increase in fibroblast cells promoted the formation of new granulation tissue. The fibroblasts then produce collagen, the main structural protein in connective tissue, which forms the framework for new tissue formation. Furthermore, fibroblasts contribute to wound contraction by pulling the wound edges together, reducing the wound area, and accelerating the healing process [29].

In K- (patch without extract), collagen fiber density was lower compared to K+ (povidone-iodine patch) due to the absence of active compounds in K-, which were essential for influencing collagen fiber density formation [30]. The K3 group (patch with 30% concentration) exhibited the highest score in collagen fiber density formation compared to other extract groups. Based on the Mann-Whitney test, the combination of red betel and green betel leaf extracts at a 30% dose showed a statistically significant difference in collagen fiber density, with a p-value of 0.046 ($p < 0.05$) compared to the K-group (group without extract). Using *Piper crocatum* at 15–45% concentrations has significantly enhanced wound healing in animal models, particularly in parameters such as inflammation reduction, granulation tissue formation, and wound area shrinkage [31]. A study demonstrated that 30% and 45% concentrations produced the most marked effects, validating 30% as a therapeutically potent and practical dose [32]. Combined with *Piper betle*, which has also shown optimal effects at similar concentrations, the 1:1 ratio used in this study at a total concentration of 30% ensures an evidence-based formulation. This approach not only captures the individual bioactivities of each extract but also enables a synergistic interaction of their phytochemical compounds in the wound-healing process [20].

A combination of plants can cause synergy effects between compounds that are greater than the use of a single plant. This is because the work of one compound can increase the therapeutic effects of other compounds, or when all compounds involved are not active themselves, they become active when combined. Plants have the potential to be synergistic due to the interactive effects of plant chemical matrices, and this is characterized by the plurality and diversity of chemical compounds that are greater than the additive effects of individual compounds. The mechanism of action of plants involves modulating biochemical pathways and changing membrane potential, receptor selectivity, and protein shift. Synergy effects can arise when compounds in one plant bind to other compounds, work together to improve the process of absorption, metabolism, and biological availability, and reduce potential side effects [33],[34].

Flavonoids functioned as inhibitors of metalloproteinase enzymes, contributing to the increased collagen content in the skin [35]. Metalloproteinase-1 (MMP-1, collagenase-1) was essential in facilitating the rapid degradation of collagen molecules in the skin [36]. In addition, flavonoids also accelerated the conversion of procollagen into collagen [37].

Tannin compounds facilitated wound healing by stabilizing collagen inducers, promoting faster tissue formation. Moreover, tannins helped minimize scar tissue development through their anti-angiogenic and antibacterial activities [38]. Saponins are another vital compound in wound healing, especially during the inflammatory phase. It enhanced the inflammatory response by stimulating vascular endothelial growth factor (VEGF) production, accelerating wound healing [39]. Saponins also impact collagen during the initial phase

of tissue repair by regulating and preventing excessive tissue formation [40]. Triterpene compounds accelerate tissue repair and wound closure by regulating ROS production in the wound microenvironment. It also stimulated cell migration, enhanced cell proliferation, and promoted collagen deposition [41]. The results of this study were consistent with previous research, indicating that applying betel leaf extract in cream form can enhance fibroblast cell count and collagen levels during the wound healing process [42]. Histopathological observations of fibroblast formation and collagen density indicated the effectiveness of the patch formulation with combined betel leaf (*Piper* sp.) extracts as a transdermal delivery system for promoting wound healing.

These findings support using transdermal patches for effective wound healing and underscore the potential of plant-based therapies in clinical practice. Further research on this transdermal patch should focus on cytotoxicity and skin irritation testing, especially for higher concentrations above 30%, to ensure safety for prolonged use. Clinical trials are needed to evaluate the systemic effects and potential interactions with other medications. Understanding the mechanism of action of bioactive compounds will optimize their therapeutic potential. At the same time, release profile studies are essential to assess how consistently and effectively the active ingredients are delivered for sustained wound healing. In addition, continuous research can be conducted with the latest innovation and technology approaches in the transdermal systems, such as the core-shell, nanoformulation-incorporated, stimuli-response microneedles, 3D-printed, and molecularly improved polymers that are in development. The development of transdermal patch preparations can be applied in the treatment of wounds, such as smart patches. Although this development approach faces various challenges, a significant effort is made to overcome these.

▪ CONCLUSION

The patch formulation containing a combination of betel leaf (*Piper* sp.) extracts shows potential as an alternative natural therapy for managing incisional wounds. The K3 group (30% extract concentration) exhibited the most effective outcomes, with a marked increase in collagen density and fibroblast cells compared to lower extract concentrations.

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