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Cocos nucifera L. endosperm promotes healing of excised wound in BALB/c mice

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ABSTRACT: Mature endosperm of *Cocos nucifera* L. (family Arecaceae) has been used in folkloric medicine as wound healing agent. However, valuable scientific evidence of its traditional use has not yet been verified. This study evaluates the wound healing activity of *C. nucifera* endosperm on the excised wound in BALB/c mice. Three concentrations (30%, 60% and 90%) of *C. nucifera* endosperm were prepared as ointment using petroleum jelly as base. The safety of the formulation was evaluated using dermal irritation test. The prepared concentrations, Solcoseryl® ointment (positive control) and petroleum jelly (negative control) were topically applied daily on the excised wound and observed for wound healing activity for 14 days, with histological evaluations at days 7 and 14. The 90% *C. nucifera* endosperm ointment showed no clinical signs of dermal irritation during the study duration, confirming the safety for topical usage of all the *C. nucifera* formulation. All groups demonstrated scab formation and wound contraction at days 7 and 9, respectively. Healing of the wounds, manifested by distinct wound contraction, was observed at day 14, with 60% *C. nucifera* endosperm exhibiting significant activity compared to the negative control (p=0.009) and untreated (p=0.046) groups. It also displayed the highest activity, with notable similarity in the activity of Solcoseryl®, and revealed the most organized epidermis comparable to normal skin. This study validates the folkloric use of *C. nucifera* endosperm in facilitating wound healing process, with 60% *C. nucifera* endosperm exhibiting the most desirable activity.

KEYWORDS: *Cocos nucifera* ; endosperm ; wound healing ; dermal irritation.

1. INTRODUCTION

A wound is defined as disruption of normal structure and function of skin and other tissues. As skin serves as an important barrier that protects our body from harmful elements in the environment, fast and efficient healing is necessary to restore its structure and function [1]. Various complications, such as dehisced wounds [2-3], hypertrophic scarring due to excessive scar formation, and contracture formation resulting to exaggeration of normal wound edge contraction forming deformities, leads to abnormal wound healing that may affect the life of patients, particularly their psychosocial aspect [4]. Although there are already available wound healing products in the market, such as topical antimicrobial and advanced dressings, negative pressure wound devices, growth factor applications, skin substitutes and hyperbaric oxygen, issues with drug resistance and side effects, and the cost of these products may hamper the effective management of wounds [5]. This problem drives the interest of many researchers to look for other sources of wound healing agents that are not only cheap and readily available, but also effective and safe to use.

Cocos nucifera L. (Family Arecaceae), commonly known as coconut, is an important fruit crop in tropical countries, recognized to be a "wonder fruit" because every part of it proves to be useful. It is a ready source of food and drink to most Filipinos and has been used in traditional medicine by many cultures. The coconut water obtained from the fruit is utilized for various health benefits such as an oral rehydration agent for diarrhea [6] and antiseptic drink for urinary tract infection while the endosperm is used for processing of different edible products [7]. Antigenotoxic effects of coconut milk, water and endosperm were also observed on bone marrow cells of experimental mice [8]. The oil from *C. nucifera* endosperm has also been used as antioxidant [9], antimicrobial [10] and wound healing agents [11].

Although there are already studies involving the use of virgin coconut oil as wound healing agent, limited knowledge on

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the utilization of *C. nucifera* endosperm as a source material for wound treatment remains. This study is advantageous as it will decrease the processes necessary for the extraction of oil, which leads to lowering of the product cost. Thus, this exploratory study is helpful in formulating a less expensive topical agent for the treatment of wounds.

2. RESULTS

2.1 Dermal irritation test

The skin areas of both male and female rabbits treated with G3 formulation displayed no clinical signs of irritation, depicted as erythema and edema. Thus, the PII values were zero, suggesting that the formulation is non-irritating. Furthermore, the skin of the rabbits on all areas tested were intact throughout the 72-h observation period.

2.2. Wound healing activity

Among the treatment groups, PC and UN groups started to display wound contraction on day 7 and 11 of the experiment, respectively (Figure 1), while the remaining groups exhibited wound contraction on day 9. All groups presented distinct wound contraction on day 14. Also, on day 14, only G2 (p=0.009) and PC (p=0.046) displayed significant wound healing activity with NC but only G2 was significantly different with UN (p=0.046). It is important to note that G2 demonstrated the highest activity comparable with that of PC. Inflammation was observed on the site of excision on day 3 of the experiment (Figure 2). Full scab formation was apparent on day 7 on all groups, following evident wound size reduction on day 9 thereafter.

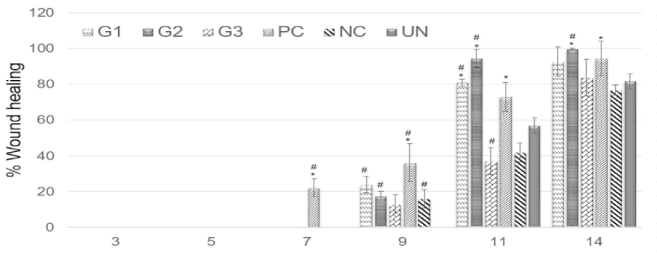

Figure 1. Percent wound healing activity of different treatment groups in excision wound model of BALB/c mice from day 0 (baseline) to day 14. 30% *C. nucifera* endosperm ointment (G1), 60% *C. nucifera* endosperm ointment (G2), 90% *C. nucifera* endosperm ointment (G3), Solcoseryl® ointment (positive control, PC), petroleum jelly (negative control, NC), untreated (UN). * – Significant difference with negative control group, # – Significant difference with untreated group.

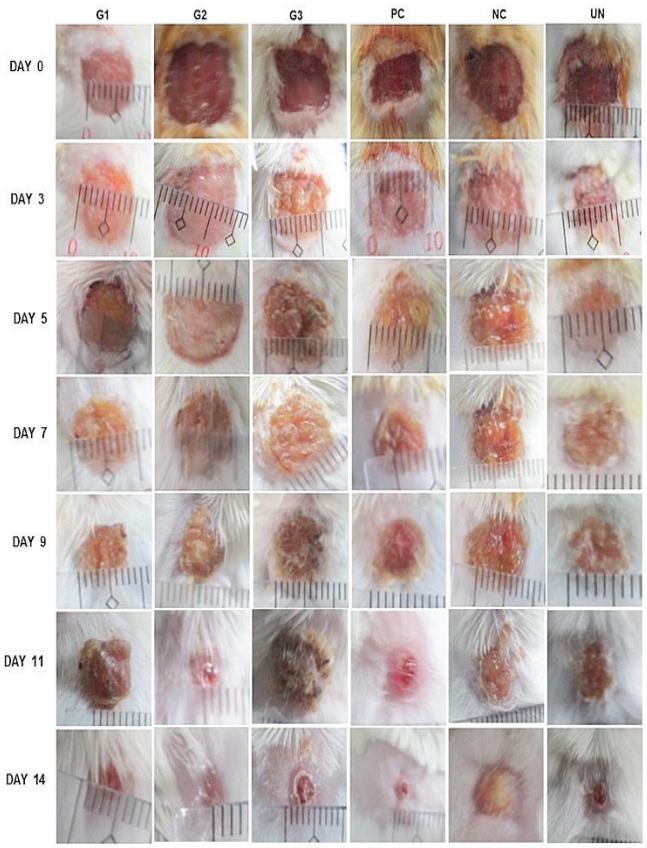

Figure 2. Wound healing progression of *C. nucifera* endosperm formulations in the excised wound generated from the BALB/c mice. 30% *C. nucifera* endosperm ointment (G1), 60% *C. nucifera* endosperm ointment (G2), 90% *C. nucifera* endosperm ointment (G3), Solcoseryl® ointment (positive control, PC), petroleum jelly (negative control, NC), untreated (UN).

2.3. Histological evaluation of healed wounds

Each group was graded individually according to the presence or absence of epithelialization, inflammatory cell infiltration, fibroblast proliferation, neovascularization and collagen deposition. Control group (C), wherein no excision was made, was used as a basis for normal skin (Figure 3).

Skin wound region of G1 were mildly infiltrated with inflammatory cells on day 7. Fibrosis was evident with increased neovascularization and enhanced proliferation of cells at day 14. Evidence of hair follicle structure development that is represented by areas of invagination of the dermis and complete re-epithelialization were also observed.

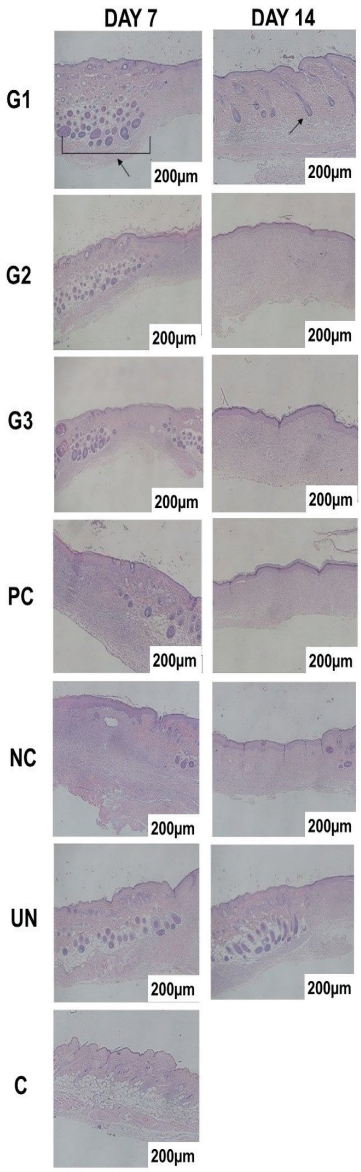

Figure 3. Histological evaluations of the healed wounds of each group after the mice were sacrificed at days 7 and 14. 30% *C. nucifera* endosperm ointment (G1), 60% *C. nucifera* endosperm ointment (G2), 90% *C. nucifera* endosperm ointment (G3), Solcoseryl® ointment (positive control, PC), petroleum jelly (negative control, NC), untreated (UN), control (C). Arrows indicate inflammatory cells (upper left) and hair follicle structure development (upper right).

For G2, most of the skin sections showed mild to moderate inflammatory reaction with increased angiogenesis at day 7. Collagen formation and fibrosis was rich ranging from mild to high. At day 14, full thickness re-epithelialization was observed in which epidermis was thin and well organized comparable to normal skin. Invagination of the dermis was also found, similar to G1.

The G3 exhibited moderate to strong inflammatory reactions at day 7 in all sections. Some sections showed complete tissue re-epithelialization at day 14 with moderate to high fibroblastic proliferation, presence of modeled dense collagen mesh, and fibrosis. A thickened keratinized layer above the dermis was also observed in some sections.

For PC, skin sections showed incomplete tissue re-epithelialization at day 7 with evident moderate to high infiltration of inflammatory cells and increased neovascularization. At day 14, moderate to high fibroblastic proliferation was evident. A thickened keratinized layer above the dermis was also observed in some sections.

At day 7, the skin sections of NC showed moderate to high infiltration of inflammatory cells and angiogenesis. At day 14, skin sections showed mild to moderate collagen formation. Inflammatory cells were also minimal.

For UN, skin sections showed incomplete tissue re-epithelialization, mild to moderate fibroblastic proliferation and presence of modeled dense collagen mesh. Mild inflammatory reactions were also observed.

3. DISCUSSION

The studies on plants that have been widely utilized in traditional medicine provide a scientific basis on their efficacy in various ailments. In this study, different concentrations of *C. nucifera* were prepared for topical application to verify its anecdotal wound healing property.

Study on dermal irritation is an essential component for the minimum set of toxicity screening, providing a fundamental characterization of the potential hazards of the *C. nucifera* endosperm ointment [12]. Rabbits were used for the dermal irritation because of its high skin sensitivity, wherein even slight skin irritant effects of a substance can be detected [13]. This model has been reported as a good predictor of skin irritancy of various substances to humans [14, 15]. In addition to rabbits, rodents have also been used but have demonstrated little capacity to respond to skin irritants [16], increasing the likelihood to have false negative results. The highest concentration was applied to increase the sensitivity and reliability of the test [17]. Based from the results, G3 showed no clinical signs of dermal irritation or corrosion, whether in the form of erythema or edema. The results corresponds to a PII value of zero, which signifies that G3 is non-irritant. The non-irritating nature of *C. nucifera* endosperm demonstrates the versatility of this product and thus, explains its traditional use for treating various diseases [6,18-19], and as food [7].

Valuable translation vehicles for human treatment modalities, which includes wound healing, are animal models [20]. In experimental wound healing assays, rodents are the most widely used animals due to their physiology that is closer to humans. Moreover, their low cost, easy handling and small

size allow a more standardized type, size, shape and depth of wound injury, small size which allows a more standardized type, size, shape, depth of wound injury, and the fact that rodents are readily available enable the study to include a large number of samples [21 - 22], which can decrease the standard error of the results. In the present study, we used the mice model to determine the wound healing activity of ointment with *C. nucifera* endosperm as the active ingredient.

Most of the literatures reported the use of *C. nucifera* oil and its processed forms as effective wound healing agent. However, limited knowledge is known on the use of the *C. nucifera* endosperm, which is easier to obtain than the oil and other related derivatives. In this study, the highest concentration of *C. nucifera* endosperm in the suitable vehicle that produced no dermal irritation was used as basis in the preparation of different doses of the ointment. Among the formulated ointments, G2 gave the highest wound healing activity at days 11 and 14. Although the study did not explore the exact mechanism of wound healing of *C. nucifera* endosperm, its activity can be attributed to antimicrobial, anti-inflammatory and antioxidant properties. The mature *C. nucifera* endosperm contains 35.5% oil which can be processed to obtain the unrefined coconut oil [23]. This oil contains high amounts of lauric acid, known to have antimicrobial activity [24], and may be one of the agents responsible for the wound healing activity. The mechanism of action of lauric acid as an antibacterial agent has been known by their ability to disrupt the cell membrane of the bacteria through penetration of the fatty acid, leading to disturbance of their enzymatic system [25]. The anti-inflammatory action of lauric acid may be attributed to the inhibition of the nuclear factor kappa B and mitogen activated protein kinase inflammatory cascades [26]. The endosperm also contains high amount of polyphenols, which are known antioxidants. These polyphenols reduce oxidative stress by decreasing the amount of free radicals produced by neutrophils and cytokines during an inflammatory response. In turn, this restores the optimum requirement for efficient wound healing [27]. Furthermore, the high percentage of wound closure observed in G2 is also supported by the collagen formation and fibrosis. Collagen formation and fibrosis iare parts of the proliferation process wherein a new extracellular matrix is developed in response to tissue injury [28]. This process promotes and establishes a good grip on the wound edges, bringing them together and therefore, closing the excised wound. This corroborated with the *in vivo* study in rodents which showed that wounded areas treated with endosperm extract healed much faster due to decreased time of complete epithelialization and higher collagen cross-linking [29].

It is noteworthy to mention that only G2 achieved comparable epidermis to normal skin and exhibited similar activity with the standard wound healing drug, Solcoseryl®. This reveals that the wound healing activity of *C. nucifera* endosperm is not dose-dependent, suggesting G2 as the most effective concentration among the formulations until day 14. Products containing *C. nucifera* oil and its derivatives are commercially available at concentrations ranging from 0.001% to 70% [30], which display the applicability of G2 for commercial use.

4. CONCLUSION

This study verifies the traditional claim that *C. nucifera* endosperm possesses pro-wound healing activity, as exhibited in excision wound BALB/c mice model. Moreover, the formulation of topical dosage form directly from the *C. nucifera* endosperm saves the hassle of additional extraction processes and cost to obtain the oil. Therefore, the formulated topical *C. nucifera* endosperm from the present study may be used as a cheaper and alternative wound healing treatment.

5. MATERIALS AND METHODS

5.1. Preparation of *C. nucifera* endosperm for topical application

Twenty (20) mature coconut seedlings were harvested from the province of Cavite, Philippines. A voucher specimen (Control number: 16-06-544) was taxonomically confirmed by Danilo Tandang of the Philippine National Herbarium Botany Division and deposited to the National Museum of the Philippines. *The samples* were cut open and the endosperm was scraped off and homogenized. Three concentrations of *C. nucifera* endosperm ointments (30%, 60% and 90%) were prepared by trituration using petroleum jelly as vehicle to produce a homogenous paste. The *C. nucifera* endosperm concentrations were stored in sterile opaque jars at 4°C prior to testing of biological activity.

5.2. Experimental animals

Healthy BALB/c mice (20–30 g, 6–8 weeks old) were purchased from the National Institutes of Health (NIH) Animal Facility and were used for the *C. nucifera* wound healing experiment. Healthy albino rabbits (1000–2000 g, 5–7 weeks old) for the dermal irritation study were procured from the University of the Philippines Los Baños. All animals were housed at the animal laboratory of NIH, kept in a room with controlled temperature (23–25°C) under 12/12 h light/dark cycle, with commercial food pellet and water supplied *ad libitum*. Acclimatization was done one week before the study, with each animal placed on separate cages. Assignment of groups to either test or to control treatments was conducted using simple randomization. All experimental procedures for the animal studies were in accordance with the guidelines of NIH and were approved by the Institute of Animal Care and Use Committee (IACUC) of the University of the Philippines Manila (approval number: 2014-021).

5.3. Dermal irritation test

The procedure was based on the Organisation for Economic Co-operation and Development (OECD) guideline 404 for acute dermal irritation test [31]. Two groups with 2 rabbits each (one male and female) were used in the study. Six (6) areas of approximately 6 cm^2, three on each side of the dorsal area of the trunk of each rabbit, were shaved 24 h before the experiment. One area was applied with 0.5 g of the *C. nucifera* endosperm ointment with the highest concentration (90%) using cotton swab and held in place with a gauze patch and non-irritating tape while the other areas were left untreated (control). After 4 h, the gauze patch was removed and the skin was rinsed with distilled water. The rabbits were observed for signs of irritation such as erythema (redness of the skin) and edema (swelling caused by accumulation of fluids) at 1, 24, 48 and 72 h after removal of residual formulation. The observations were graded using the OECD scoring system (Table 1). The primary irritation index (PII), which is the quantitative measurement of the acute toxic effect of substances on skin as depicted by erythema and edema, was calculated using equation 1[32]:

(Eq. 1)

$$PII = \frac{\Sigma Erythema + \Sigma Edema\ (at\ 1,24\ 1\ 48\ and\ 72\ h)}{No.of\ test\ sites} x\ 4\ scoring\ intervals$$

5.4. Wound healing activity

The method for excision wound creation was adapted from Mughrabi *et al.* [33] with slight modifications. Thirty healthy BALB/c mice of either sex were randomly divided into 6 groups (G1 – 30% *C. nucifera* ointment; G2 – 60% *C. nucifera* ointment; G3 – 90% *C. nucifera* ointment; PC – Solcoseryl® as positive control; NC – petroleum jelly as negative control; and UN – untreated). The animals were anesthetized with 0.1 mL of Tiletamine/Zolazepam (Zoletil®) IM prior to shaving and disinfecting with 70% alcohol. The dorsal area were marked, and wounds were then created along the markings and left undressed. The wound area, ranging from 0.5-2.25 cm^2, was measured immediately using a ruler.

Mice received 0.25 g of the samples per day except for UN, which was given no treatment. Treatments were given topically to wounded animals once daily for 14 days. Percent wound contraction, which is the healing manifestation of wound, were measured six times (days 3, 5, 7, 9, 11 and 14) to monitor the progression of wound healing. Percentage wound closure was calculated using equation 2 [34]:

(Eq. 2)

$$\%\ Wound\ closure = \frac{Initial\ area\ of\ wound - nth\ day\ wound\ area}{Initial\ area\ of\ wound} x\ 100$$

Table 1. Grading of skin reactions.

Erythema and eschar formation		Edema formation	
No erythema	0	No edema	0
Very slight erythema (barely perceptible)	1	Very slight edema	1
Well defined erythema	2	Slight edema (edges of area well defined by definite rating)	2
Moderate to severe erythema	3	Moderate edema (raised approximately 1 mm)	3
Severe erythema (beef redness) to eschar formation preventing grading of erythema	4	Severe edema (raised more than 1 mm and extending beyond area of exposure)	4

5.5. Histological evaluation of healed wounds

On day 7, two mice were sacrificed for each group. Skin tissue samples were immediately fixed after animal dissection with 10% formalin, dehydrated in graded series of alcohol, cut in 2 µm thick and stained with hematoxylin and eosin (H&E). Additional sections were stained with Masson's trichome (MT) to evaluate collagen formation. Same procedure was also employed on the remaining mice after day 14. The tissue examination was performed by a veterinary pathologist using a compound light microscope. Samples were assessed blindly for epithelialization, inflammatory cell infiltration, fibroblast proliferation, neovascularization and collagen deposition. Stained sections were graded in a blind fashion using the modified 0 to 4 numerical scale (Table 2) [35].

Table 2. Grading system for histological evaluations.

0	Absent
1	Mild presence
2	Moderate presence
3	Strong presence

5.6. Statistical analysis

The data gathered were expressed as Mean ± standard error of the mean (SEM). Statistical significance of differences between groups and control was determined by one-way analysis of variance (ANOVA), followed by Tukey's *post hoc*

test using SPSS 17.0 software. Mean values were considered statistically significant when $p<0.05$.

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Authorship statement

Author contributions: Concept – E.C.A.; Design – E.C.A., A.A.S.; Supervision – E.C.A., A.A.S.; Materials and Resource– J.D.A.; Data Collection and/or Processing – R.B.C., L.B.T., K.M.G.P., R.A.M.M., G.L.T.D.; Analysis and/or Interpretation – R.B.C., L.B.T., K.M.G.P., R.A.M.M., G.L.T.D.; Literature Search – K.M.G.P., R.A.M.M., G.L.T.D.; Writing – R.B.C., K.M.G.P., R.A.M.M., G.L.T.D.; Critical Reviews – E.C.A., A.A.S., J.D.A., R.B.C.

Conflict of interest statement

The authors declared no conflict of interest.

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