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Evaluation of Wound Closure Activity of Cocos Nucifera Oil on Scratched Monolayer of Human Dermal Fibroblasts

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Cocos nucifera L. has been widely used in Malaysia for traditional skin moisturiser and as a food supplement for centuries. Virgin coconut oil (VCO) contains predominantly medium chain fatty acids of which 45 - 50 % is lauric acid (C12). VCO has also been reported to contain antioxidants and polyphenols. In this study, integrated wet extracts of VCO was investigated for its efficacy on cell viability and migration in fibroblast cells (HSF 1184). The in-vitro wound healing assay was developed at a density of 3 × 105 cells/well in 6-wells plate to 80 % confluence. A straight line scratch was performed using a sterile 200 µL pipette tip to create a wound on the monolayer culture. The treatment medium of VCO (0.1 - 1.0 mg/mL), ascorbic acid (5 µg/mL) (positive control) and serum-free media (control) were filled in the 6-wells culture plate. Wound closure image at the same location was taken under inverted microscope at various intervals (0 h, 6 h, 12 h, 24 h). The percentage of wound closure was calculated and analysed using Image J software. As a result, the percentage of wound closure for HSF 1184 using VCO extract 1.0 mg/mL was about 56.89 ± 2.35 % comparable to ascorbic acid (55.65 ± 1.08 %) after 24 h of treatment time. The wound closure for VCO 0.5 mg/mL was about 60.51 ± 3.22 % and VCO 0.1 mg/mL (61.66 ± 3.62 %) after 24 h. In conclusion, VCO contains antioxidants and phenolic compounds that might promote the proliferation and migration of cells to enhance wound closure activity on human dermal fibroblast cells.

1. Introduction

Coconut oil has long been used by some ethnic groups of Ngada in Flores, an eastern Indonesian island, who practise the Ayurvedic system of medicine for various skin disorders including wound healing, microbial infections and to preserve medicinal plants (Sachs et al., 2002). Virgin coconut oil (VCO) obtained using the wet extraction process from fresh coconut milk has better antioxidant properties and phenolic content compared to coconut oil obtained from the drying process (Marina et al., 2009a). Marina et al. (2009a) has also reported that VCO contains the highest phenolic content as well as the highest antioxidant activity. The method of wet extraction can retain the bioactive components in VCO such as polyphenols and vitamin E compared to copra oil (Nevin and Rajamohan, 2006).

Polyphenols has the ability to inhibit free radical scavenging activities (Bito et al., 2000). The high polyphenol content in coconut oil is capable of maintaining normal levels of lipid parameters in tissues and the serum helps by trapping reactive oxygen species in aqueous components such as plasma and interstitial fluid of the arterial wall (DebMandal and Mandal, 2011). VCO contains adequate amounts of nutrients such as vitamin E and A to improve wound healing activities (Nevin and Rajamohan, 2010). Vitamin A is required for cellular differentiation, immune function, epithelial and bone formation (Petry, 1996). Vitamin E is the major lipid-soluble antioxidant in the skin. Topical application of vitamin E is beneficial for speeding wound healing and improving cosmetic outcome of burns and other wounds, including surgical scars (Baumann and Spencer, 1999).

In the previous study, the topical application showed an effect of VCO onto human skin disorder such as Atopic Dermatitis (AD), which has shown superior respond compared to mineral oil in term of SCORAD (SCORing of Atopic Dermatitis) index values, transepidermal water loss (TEWL), and skin capacitance

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(Therese et al., 2014). VCO is safer and cheaper compared to synthetic drug because it is made of natural substances from plant sources. VCO is possible to be used as substitute for steroidal and non-steroidal wound healing drugs to treat the wounded skin. There is limited scientific research about the benefits of VCO from the point of skin barrier protection and skin treatment. The effect of VCO usage on human skin cellular model needs to be explored further. In this study, VCO was used to study the effect of bioactive compounds such as phenolic contents and antioxidants present in the VCO toward the in vitro wound closure activity. The in vitro wound healing assay was used to examine the cell migration and proliferation activity of human dermal fibroblast cells.

2. Methodology

2.1 Material and chemicals

Coconut fruits known as Cocos nucifera were selected from the species of West African Tall (WAT). The harvesting cycle of the coconut from one specific location (Department of Agriculture, Batu Pahat, Johor) was recorded at 120 d. The fresh coconut milk was processed by local coconut suppliers in Gelang Patah, Johor. The reagent and chemicals used in the analysis of phenolic content and antioxidant were from Folin and Ciocalteu's phenol, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and sodium carbonate (Sigma-Aldrich Chemie GmbH, Steinheim). The chemical used in the cell culture assay were from Dulbecco's modified essential medium (DMEM), 10 % fetal bovine serum (FBS), 1 % of penicillin-streptomycin antibiotics, trypsin-EDTA 0.25 % and phosphate buffer saline (PBS) from Gibco, Life Technologies, USA, dimethyl sulfoxide (DMSO) and (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) MTT solution from Sigma Aldrich Chemie GmbH, Steinheim.

2.2 Extraction of VCO method

The extraction of VCO using integrated wet process (IWP) was performed according to the method proposed by Nur Arbainah (2012) with slight modifications. The solid endosperm of mature coconut was de-husked, grated, and made into viscous slurry. The slurry was squeezed through cheesecloth to obtain coconut milk and refrigerated for 24 h. Later, the coconut milk was stirred using a mixer until coconut butter was formed. After that, the water was separated from the coconut butter. Then, the coconut butter was soaked in a water bath (Memmert, USA) at 37 °C. This step was followed by the centrifugation of the solution to separate oil from the aqueous layer. The obtained VCO was filtered through cheesecloth and kept in storage at room temperature prior to use in the next experiment (Ahmad et al., 2015).

2.3 Analysis of bioactive compound in VCO extract

2.3.1 Total phenolic content

Polyphenol (PF) from the test oils was extracted according to the method described by Gutfinger (1981). 10 g oil was dissolved in 50 mL hexane and extracted three times with 20 mL portions of 60 % methanol (Qrec (Asia) Sdn. Bhd., Malaysia) successively. Triplicates of extracts and solvents were evaporated to dryness (60 °C) using a rotary evaporator (Laborota 4003, Heidolph, Germany). The final residue was mixed in a known volume of 60 % methanol. The total polyphenol content of this solution was estimated using Folin– Ciocalteau reagent. An aliquot of test samples (1 mg/mL) was mixed with 1.0 mL of Folin-Ciocalteau reagent (previously, it was diluted with 10-fold of DI water). A sodium carbonate solution (0.8 mL) of 7.5 % was added and allowed to stand at room temperature for 30 min. The absorbance of the phenolic content in methanol was read using a spectrophotometer (Shimadzu UV–1800, Japan) at 765 nm. The total phenolic content was expressed as gallic acid equivalents per 100 g oil (Nevin and Rajamohan, 2006).

2.3.2 Antioxidant activity

DPPH radical-scavenging activity of the phenolic extracts of VCO was measured according to the method reported by Hatano et al. (1988). The phenolic extracts were adjusted to the required concentrations through suitable dilutions with the same solvent system used for the extraction of phenolic substances. Each phenolic extract (300 µL) of various concentrations (25 mg/L, 50 mg/L, 75 mg/L and 100 mg/L) from VCO was added to a methanolic solution of DPPH (0.3 mL, 0.8 mM) and the resultant mixture was swirled at 40 Hz for 5 min. After 30 min of incubation in the dark, the absorbance of each reaction mixture was measured at 517 nm using a spectrophotometer (Shimadzu, UV–1800, Japan). The DPPH radical scavenging activity was measured similarly for a series of 25 – 100 mg/L solutions of α-Tocopherol as a positive control (Marina et al., 2009b). The inhibitory effect of DPPH radical was calculated according to Eq(1):

$$\text{Inhibition (\%)} = [(A_0 - A_1 / A_0)] \times 100\ \% \quad (1)$$

Where A_0 is the absorbance of the reaction mixture with solvent system (control) and A_1 is the absorbance of the reaction mixture with phenolic extract or α-Tocopherol (Marina et al., 2009b).

2.4 Biological assay in cell culture

2.4.1 Cell viability assay

Human skin fibroblast cells (HSF 1184) was grown at a density of 1×10^5 cells/well in a 96-well plate and cultured in 150 µg/mL/well DMEM for 24 h to allow the cell to confluence in the well. PBS was filled after removal of the medium. The cells were cultured in serum-free DMEM (control), VCO and ascorbic acid, 5 µg/mL (positive control) for 24 h. The VCO was tested using various concentrations from 1.0 mg/mL to 10.0 mg/mL. Following the treatment with the VCO, the culture medium was added and incubated with MTT solution (a solution of 5 mg/mL in PBS) about 10 µL in each well at 37 °C for 3 - 4 h. The solution was discarded and replaced with 150 µL DMSO in each well. After 30 min of incubation, the absorbance of the supernatant was measured at 570 nm using a microplate reader (Erba Lisa Scan 11, Mannheim) (Hasham et al., 2014). The cell viability was calculated using Eq(2):

$$\text{Cell Viability}\ (\%) = (\text{Sample}/\text{Control}) \times 100\ \% \quad (2)$$

2.4.2 In vitro wound closure activity

HSF 1184 was used in this scratch wound closure assay to observe the migration capability of the cells wound closure. The cells were developed at a density of 3×10^5 cells/well in 6-wells plate to 80 % confluence. A straight line scratch was cut with a sterile 200 µL pipette tip (yellow tip; 2 - 200 µL, 53 mm) to create a 0.4 mm to 0.5 mm width wound on the monolayer culture. Cell debris was washed with PBS twice and replaced with 3.0 mL extract of VCO with concentrations from 0.1 mg/mL, 0.5 mg/mL and 1.0 mg/mL, serum-free DMEM media (control) and ascorbic acid, 5 µg/mL (positive control). All experiments were done in triplicate. Wound closure at the same location was observed under inverted microscope at various intervals (0 h, 6 h, 12 h, 24 h) and digitised images were taken. The wound closure was analysed using Image J software (Mustaffaa et al., 2015). The percentage of wound closure was calculated using Eq(3):

$$\%\ \text{Wound closure} = (W_{0h} - W_{Xh}) / W_{Xh} \times 100\ \% \quad (3)$$

Where,
W_{0h} = Wound at 0 h
W_{Xh} = Wound at 'x' h ('x' = 0 h, 6 h, 12 h and 24 h)

2.5 Statistical analysis

All results were presented as the average ± standard error of mean (SEM) of the combined data from replicate experiments. The analysis for wound closure was performed using Image J software (National Institute of Health, 1997).

3. Results and discussion

3.1 Total phenolic content and antioxidant compound in VCO extract

The analysis of bioactive compounds such as total phenolic content and antioxidant was carried out in a previous study by Ahmad et al. (2015). The total phenolic content and antioxidant compound in the VCO extract was obtained from an integrated wet process (IWP) of 16.02 ± 0.44 mg GAE / 100 g oil and 5.07 ± 0.19 mg/L (Table 1). Marina et al. (2009b) revealed that VCO extracted through a wet process has high phenolic contents. The antioxidant activity in VCO extract also has high radical scavenging activities, comparable to α-tocopherol (Marina et al., 2009b). The study suggested that the phenolic compound in VCO could contribute to the antioxidant activity (Marina et al., 2009b). According to Nevin and Rajamohan (2010), the antioxidant compound in VCO extract could have a significant effect on cutaneous wound healing in animals which were treated. VCO also contained minor component such as polyphenols, vitamin E and pro-vitamin A with significant radical scavenging activities to improve the wound healing process (Nevin and Rajamohan, 2010).

3.2 Effect of VCO extract on the cell viability

Cells from the fibroblast cell line (HSF 1184) were exposed to increasing concentrations of VCO extracts for 24 h and their viability was assessed using the MTT assay. The results are presented in Figure 1.

Table 1: The phenolic content and antioxidant properties of VCO extract (Ahmad et al., 2015)

Parameter	Value
a) Polyphenol (mg GAE / 100 g oil)	16.02 ± 0.44
b) Antioxidant free radical scavenging activity, EC50 (mg/L)	5.07 ± 0.19
c) α-Tocopherol free radical scavenging activity (positive control), EC50 (mg/L)	4.16 ± 0.50

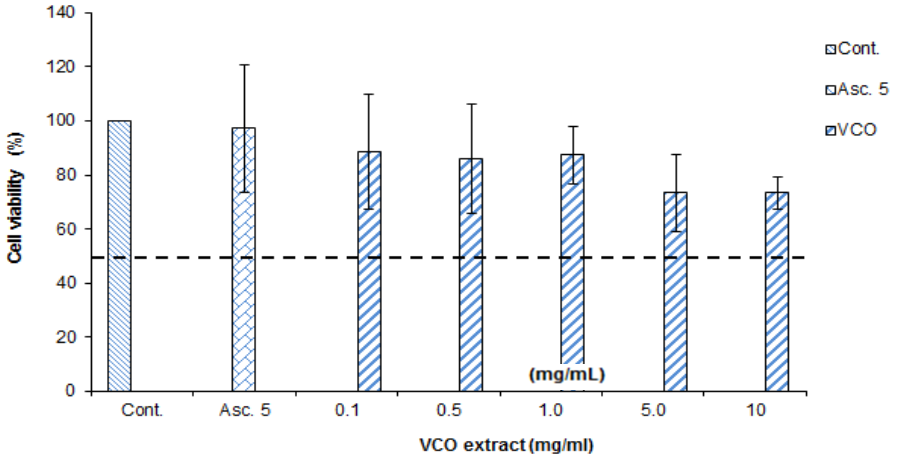

Figure 1: Cell viability of fibroblast cells (HSF 1184) cultured with increasing concentrations of VCO extract using MTT assay. Data are presented as mean ± SD (n=3). (Cont. represents control, Asc. 5 represents ascorbic acid)

The data showed that the cytotoxicity of VCO was determined by their concentrations. It was observed that the percentage of cell viability was achieved above 50 % within 0.1 - 10.0 mg/mL of VCO concentrations. The viability assay using VCO extract was very effective at low doses; a more robust effect was noted for fibroblasts proliferation. Higher concentrations of VCO (100 mg/mL) were cytotoxic and caused a significant decrease of cell viability to 28.9 % (data not shown). This result showed that the VCO extract increased cell growth of the fibroblast cell lines but was still lower than ascorbic acid (as positive control) with 88 % of cell viability. Based on the results, VCO extract was able to enhance the proliferation and viability of human dermal fibroblast cells.

3.3 Effect of VCO extract on fibroblast cells in wound scratch assay

The ability of VCO extract to stimulate fibroblast proliferation and cell migration in enhancing wound healing activity was demonstrated using in vitro wound scratch test. A confluent monolayer of fibroblast cells was scratched to generate the wound healing model using a sterilised pipette tip. Then, the wounded gap was treated using different concentrations of VCO extracts, ascorbic acid and serum-free media as control. The fibroblast cells were allowed to proliferate and migrate from 0 h to 24 h at 37 °C. The scratch test result is shown in Figure 2.

The fibroblast cells migration was observed after 6 h of treatment and scratch closure after 24 h was about 56.89 ± 2.35 % compared to untreated control using VCO extract 1.0 mg/mL comparable to ascorbic acid 55.65 ± 1.08 % (Figure 2). The VCO extract (1.0 mg/mL) exhibited the highest percentage of wound closure compared to other VCO concentrations. For the untreated cells, the cell migration was slower and the wound closure was about 67.84 ± 2.80 % after 24 h of treatment. The VCO extract contains bioactive compounds such as phenolic compounds and antioxidants that induce fibroblast cell growth and proliferation to enhance the wound closure activity. If higher concentrations of VCO extract were used in the scratch assay, more phenolic and antioxidant compounds would be available to promote cell proliferation and closure rate. The wound healing effect of coconut oil is influenced by antioxidant and antibacterial properties present in the oil (Wilson et al., 2015). However, the in vitro scratch assay could not show the wound closure activity similar to actual wounds such as cutaneous and burn wounds. The in vivo cutaneous wound healing was studied using animal models, for example, rats, to study the complex process of wound healing mechanisms treated with coconut oil (Wilson et al., 2015). Medium chain fatty acids (MCFA), the major component of VCO, which is lauric acid (45 - 50 %) could contribute to stimulate the migration and proliferation of fibroblast cell. Fatty acids

have been proven to promote cellular proliferation (Rose et al., 1994) and growth factor activities (Jiang et al., 1995).

Cell migration and proliferation have rate limiting factors in skin regeneration (Walter et al., 2010). Upon the creation of a wound, cells start to migrate at the edge of the rupture (Burk, 1973). Individual cells start to spread at the wound's edge followed by cell migration (translocation) and cell proliferation (Coomber and Gotlieb, 1990). The antioxidant and anti-inflammatory properties could help to protect the wound area from bacterial infection, reduced inflammation and induced cell proliferation to support the reconstruction of damaged tissue (Kulac et al., 2013).

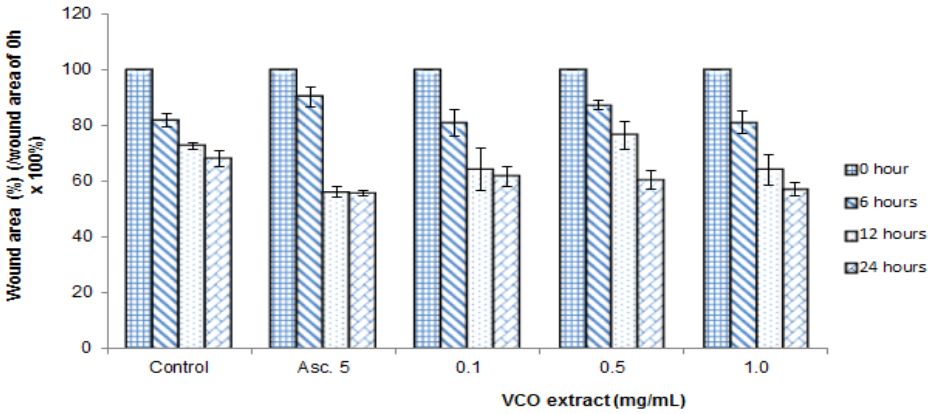

Figure 2: The effect of various concentr ations of VCO extract (mg/mL) on the percentage of wound closure (%) of scratched fibroblast cells; ascorbic acid indicated by Asc. 5.

4. Conclusion

This study has demonstrated the activity of the VCO extracts in enhancing proliferation and viability of human dermal fibroblast cells in in vitro wound closure assay. The VCO extract contains phytochemicals such as phenolic compounds and also shows high antioxidants activity that might induce fibroblast cell growth and proliferation to enhance the wound closure activity. The optimum VCO concentration obtained in this study was 1.0 mg/mL to give the highest wound closure about 56.89 ± 2.35 % after 24 h. These findings could lead to the drug discovery of wound healing active agents from plant-based natural compounds. Further preclinical investigations need to be conducted in future research.

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