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Wound Healing Activities of Hydrolyzed Virgin Coconut Oil (HVCO) and Fucoidan Combination: An In Vitro Assay

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ABSTRACT

Objective: The aim of this study was to investigate the wound healing activity of hydrolyzed virgin coconut oil (HVCO) and fucoidan combination, in the NIH 3T3 cell line using in vitro assay, and compared with HVCO and fucoidan alone.
Methods: NIH 3T3 Cell proliferation was assessed using the MTT method, migration activity was assessed using sctrach wound healing assays and expression of COX-2 and VEGF protein were determined using immunocytochemistry (ICC).
Results: The results from the proliferative activity assay showed that the best concentrations for all samples were 31.25 µg /ml. NIH 3T3 cells migration activity assay showed that the best combination of the HVCO and fucoidan was 50:50.From COX 2 and VEGF protein expression test results, the combination of HVCO and fucoidan has a higher percentage of expression than HVCO or fucoidan alone.
Conclusion: The results reveal that the combination of HVCO and fucoidan had better wound healing activity than HVCO or fucoidan alone.

Keywords: MTT, Proliferation, Migration, Immunocytochemistry

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INTRODUCTION

Wounds can be defined as the occurrence of damage to living tissue or the rupture of the integrity of the epithelium of the upper layer of the skin. Human skin is the largest organ in the body that covers the outer layer, and is divided into three main layers namely the epidermis, dermis, and hypodermis (sub cutaneous). The skin acts as a guard for internal organs, providing protection from microbes, regulating body temperature, and as a place for touch, heat and cold receptors[1]. Wound Healing is a natural phenomenon, occurs by replacing devitalized and missing cellular structure and tissue layers of the skin, this process consists of four highly integrated and overlapping phases: hemostasis, inflammation, proliferation, and tissue remodeling orresolution[2,3].

Virgin coconut oil (VCO) contains mainly medium chain fatty acids especially lauric acid, easily absorbed, has the potential to accelerate cell metabolism and moisten wound. Previous research has shown that Hydrolyzed virgin coconut oil (HVCO) biological activity is better than virgin coconut oil (VCO) [4]. Previous research also has proven the potential of angiogenesis and wound healing from fermented virgin coconut oil, both in vivo and in vitro[5].Fucoidans are a family of sulphated polyfucose polysaccharides, have attracted considerable biotechnological research interest since the discovery that they possessed anti-coagulant activities similar to those of heparin. Fucoidan are also reported to possess other properties including antithrombotic, anti- adhesive and anti-viral effects. Many of these effects are thought to be due to their interactions with growth factors such as basic fibroblast growth factor (bFGF) and transforming growth factor-b (TGF-b). Fucoidans may, there fore, be able to modulate growth factor-dependent pathways in the cell biology of tissue repair[6].

The purpose of this study was to determine wound healing activities from HVCO in combination with fucoidan in NIH 3T3 cell culture with in vitro assay, and compared with HVCO and fucoidan alone. Cell proliferation activity

were carried out by the MTT method, while the cell migration activity was carried out using the scratch migration assay method. Protein expression activity that regulates the process of angiogenesis (COX-2 and VEGF) using the immunocytochemistry method.

MATERIALS AND METHODS

MATERIALS

Hydrolyzed Virgin Coconut Oil (HVCO), Fucoidan (PT. Kalbe Farma Tbk., Indonesia), Aloclair® Plus Gel (PT. Kalbe Farma Tbk, Indonesia) as a positive control, Dulbecco's Modified Eagle medium (DMEM) complete medium, Fetal Bovine Serum (FBS) 10% (v/v) (Gibco), penicillin–streptomisin 2% (v/v) (Gibco), dan Fungizon (amfoterisin B) 0,5%. 0,25 % tripsin-EDTA (Gibco), Phospate Buffer Saline (PBS), MTT (3-(4,5-dimetiltiazol-2-il)-2,5 difeniltetrazolium bromide) (Sigma),Sodium Dodesil Sulphatet (SDS), beta mercapthoethanol, ethanol 70%, HCl 0,1 N (Merck)

METHODS

NIH 3T3 Cell proliferation activity assay

This procedure was purpose to determine the best concentration of sampels. 10,000 cells/well in DMEM complete medium were seeded into 96-well plates, incubate in a humidified atmosphere (5% CO_2) at37°C. After 24hrs, growth medium DMEM complete medium was replaced with medium containing different concentrations of HVCO, fucoidan and positive control. Subsequent to incubation for 24hrs, cells proliferation activitywas determined by MTT assay. Briefly, 100 ml of medium containing 10 ml MTT (5 mg/ml) was added into each well and incubated for 4h. The MTT reaction was stopped with a reagent stopper (10% SDS in 0.01N HCl), the wrapped plate was left for one night in room temperature. Uptake is read by a Microplate Reader (Biorad) at a wavelength of 595 nm. Tests are carried out with three repetitions for each sample. The data which were absorbed from each well were converted to percentage of viable cells[7]. The concentration of the sampels with the highest percentage of cells proliferation activitywere the best concentration for the samples.

Cell Migration activity Assay

The migration activity was aim to determine the combination with the best activity, a series of HVCO and fucoidan concentrations were made in 3 series with a comparison between HVCO and Fucoidan with a ratio of 50:50; 75:25; 25:75 percent of each effective concentration value. The method used is the scratch wound assay. NIH 3T3 cells were seeded in complete medium DMEM at $5x10^4$cells/well in 24-well plates and incubated for 24 h at 37°C. Cultured cells were washed with PBS and added culture media which containing 0.5% FBS and incubated for 24 h. Scratch was done in the bottom center of the well within cell layer using yellow tip. Residues cell in the plate were washed with PBS and treated with EAF and incubated for 72 h at 37°C and documented under inverted microscope against cell migration rapidity after 0, 24, 48 and 72hrs. The space from scratch treatment between control and treatment cultur cell was quantified using Image J software and defined as cell migration area. The combination with the best activity was used in protein expression of COX-2 and VEGF assay.

Expression of COX-2 and VEGF proteins activity assay

Examination of COX-2 and VEGF proteins was carried out at the best combination concentration carried out using the immunocytochemistry (ICC) method. $5x10^4$ cells /wells of NIH 3T3 cell cultures were transferred into a 24-well plate filled with a cover slip, then the cells were incubated overnight at 37°C in a 5% CO_2 incubator. After the cells recover, they are treated by giving the test material and re-incubating for one night. At the end of the incubation time, PBS cells were washed and then cold methanol was added and incubated at room temperature for 10 minutes. The fixed cells were then washed with distilled water 2 times and then incubated in a hydrogen peroxidase solution for 10 minutes. Furthermore, cells were dripped with prediluted serum blocking and incubated 10 minutes. Then it was dropped with primary Monoclonal antibodies for COX-2 and VEGF (1:50 dilution), incubated for 10 minutes, and washed in PBS 3 times. The preparations were incubated in biotin for 10 minutes and washed with PBS 2 times for 5 minutes. After washing with PBS, the cell is dripped with secondary antibodies (biotinylated universal secondary antibody) and injected back for 10 minutes. Then the preparations were incubated in streptavidin-peroxidase for 10 minutes and washed with PBS twice for 5 minutes. Next, the preparations were washed again with PBS and then incubated in DAB for 10 minutes and washed with distilled water. The preparation was then immersed in Mayer-Haematoxylin solution for 3-4 minutes for counterstain and washed with distilled water. The slip cover is then lifted and dipped in xylol, then dipped in alcohol. After drying, the slip cover is placed on the glass object and pressed with glue (mounting media). Cover slip is covered with a slide then an observation with a microscope. Observations were carried out by microscopes equipped with optical fiber, each dosage was observed in five fields of view with even distribution. The documentation results were then analyzed by using Software Optilab Viewer Image Raster to saw the number of cells expressing the protein.

Statistical analysis

Normality of the data was performed with the Shapiro-Wilktest. Statistical analysis was performed by the one-way ANOVA with tukey test for homogenous subsets ($\alpha < 0, 05$).

RESULT & DISCUSSION

NIH 3T3 Cells proliferative activity

MTT method was used to determine cell proliferation activity after incubation for 24 hours. The result of percentage of cell Proliferation at various concentrations can be seen in Table 1. Cell control was assumed to have a percentage of live cell counts of 100%. The highest percentage of living cells for the results of HVCO was at a concentration of 31.25µg/ml, this concentration was considered as the best concentration for the HVCO. For the fucoidan, the highest percentage of living cells was found at a concentration of 31.25µg/ml, this concentration was also considered the best concentration for the fucoidan. As for positive control (Aloclair®), the highest

percentage of living cells at concentration of 125µg/ml, but this result was not much different from the sample concentration of 31.25µg/ml. Therefore the concentration of 31.25µg/ml was considered to be the best concentration, because with smaller doses or concentrations it can produce almost the same effect with larger doses. From the data above, the results of best concentrations for all samples were 31.25µg /ml.

Table 1: NIH 3T3 cells proliferation activity at various concentrations

Sampels	Concentration	Cells proliferation percentage (%)
HVCO	500 µg/ml	10,8333±0,55120*
	250 µg/ml	26,7363±3,75075*
	125 µg/ml	129,5833±5,63027*
	62,5 µg/ml	133,7500±0,24076*
	31,25 µg/ml	139,4443±1,52311*
	15,625 µg/ml	89,0377±0,57783
Fucoidan	500 µg/ml	106,5280±4,98008
	250 µg/ml	109,8610±1,99221
	125 µg/ml	113,1943±8,57484
	62,5 µg/ml	121,2503±4,92566
	31,25 µg/ml	121,1807±8,94639
	15,625 µg/ml	89,0377±0,49661
Aloclair®	500 µg/ml	127,2917±1,57728*
	250 µg/ml	156,2503±4,63980*
	125 µg/ml	156,7360±5,30664*
	62,5 µg/ml	152,1527±3,95362*
	31,25 µg/ml	145,6250±7,55294*
	15,625 µg/ml	88,3540±0,54317*
Cells control		100,0000±5,92664
Medium control		0,0000±4,82857*

Values are expressed as mean± SEM using one way ANOVA; n =3 in each group
*p<0, 05 : significantly different with cell controle

NIH 3T3 cells migration activity

The migration method is used to measure the percentage of wound closure by the test material. Migration is a key property of live cells and critical for normal development, immune response[8]. Cell migration is a complex phenomenon related to many cellular processes. Migration testing was done by scraping monolayer cells using sterile yellow tips until a certain size of stroke was formed. Determination of cell migration ability was done by quantifying the width of the stroke at 0 hour and at certain time intervals until the cells migrate to cover the scratches. This measurement directly allows to know the effect of a compound with different concentrations on the ability of cell interactions during cell migration. From the results of calculating the percentage of wound closure, which was calculated based on the initial empty area compared to the area after incubation in Image J software, the data in Table 2 is obtained. Wound healing activities from the sampels based on NIH 3T3 cells migration assay can be seen in Figure 1.

Table 2: Percentage of wound closure from the test material by NIH 3T3 cell migration testing with the incubation period of 24 hours, 48 hours and 72 hours.

S. No	Sampels	Percentage of wound closure (%)		
		24 hours	48 hours	72 hours
1	Cells control	15,2433±0,41478	74,3600±0,45574	100,0000±0,0000
2	Aloclair®	28,5767±0,45157*	82,5467±0,91732*	92,7167±0,54272*
3	Fucoidan	29,7100±0,58158*	85,6167±0,30322*	100,0000±0,0000*
4	HVCO	62,3700±0,44736*	100,0000±0,0000*	100,0000±0,0000*
5	50:50HVCO Fucoidan combination	77,5067±0,28620*	100,0000±0,0000*	100,0000±0,0000*
6	25:75 HVCO Fucoidan combination	2,5433±0,16496*	-3,3267±0,20755*	-18,2367±0,02404*
7	75:25 HVCO Fucoidan combination	-9,0700±0,35346*	-18,4765±0,02404*	-29,2733±0,29157*

Values are expressed as mean± SEM using one way ANOVA; n =3 in each group
*p<0, 05: significantly different with cell control

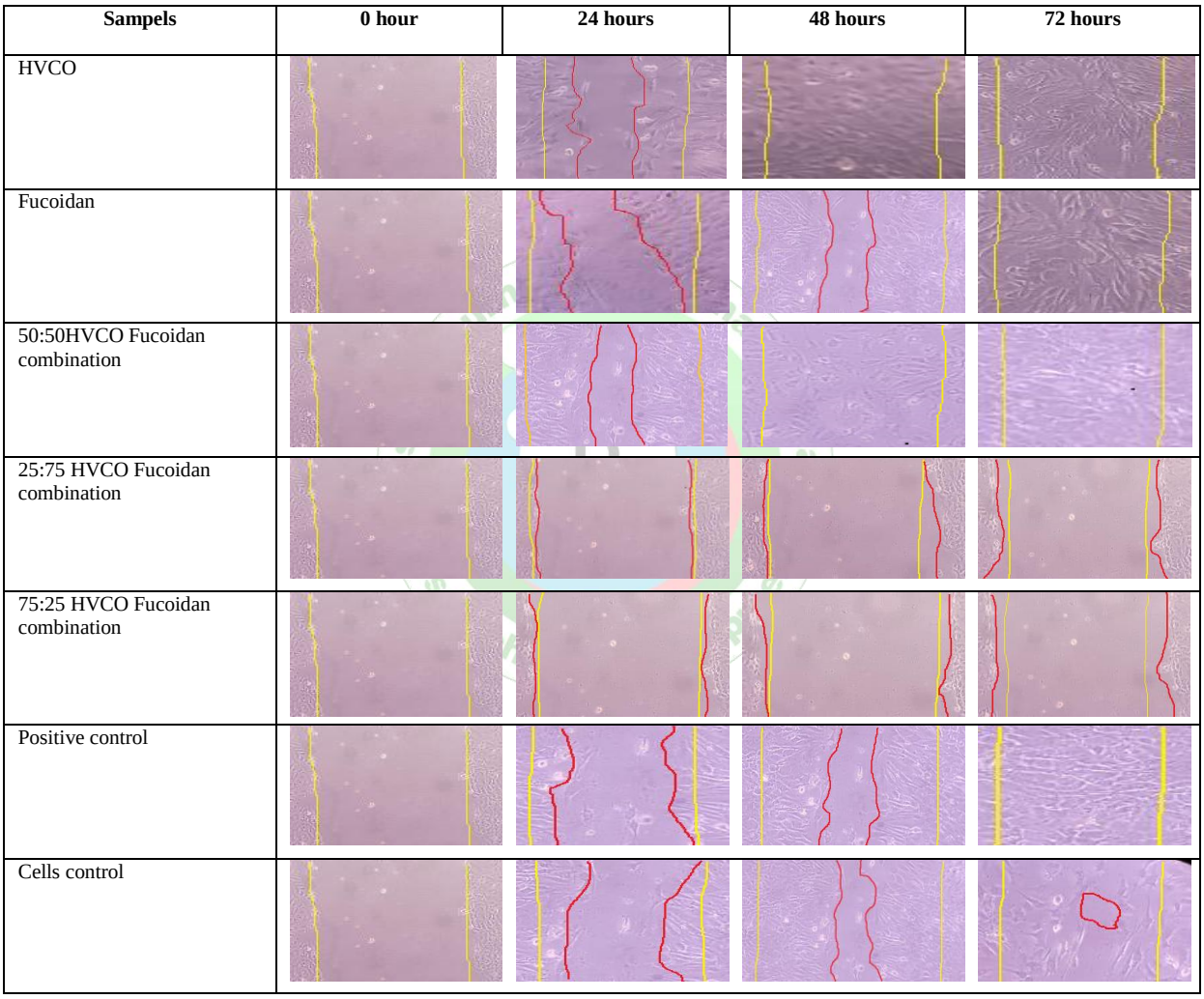

Figure 1: Documentation of the wound closure process of the samples at 0 hours, 24 hours, 48 hours and 72 hours.

The results of NIH 3T3 cells migration activity assay showed that the best combination of the HVCO and fucoidan was 50:50, so that the best combination would be used for further eksperiment of protein COX-2 and VEGF expression activity by immunocytochemistry (ICC) method.

Expression of COX-2 and VEGF protein activity assay

Immunocytochemistry (ICC) is a method that used to detect the expression of a specific protein or antigen in cells using specific antibodies that will bind to proteins or antigens. Data obtained in this test in the form of a percentage was the percentage comparison of cells expressing proteins from the total number of cells. Data analysis was performed to calculate the number of cells expressing COX-2 and VEGF proteins using Optilab Viewer 2.2 Image Raster software. The results of the analysis are through image quantification from an average of 5 (five) fields of view. Cells that are brown/ dark cell cytoplasm mean expressing the protein, while blue cells mean not expressing protein.

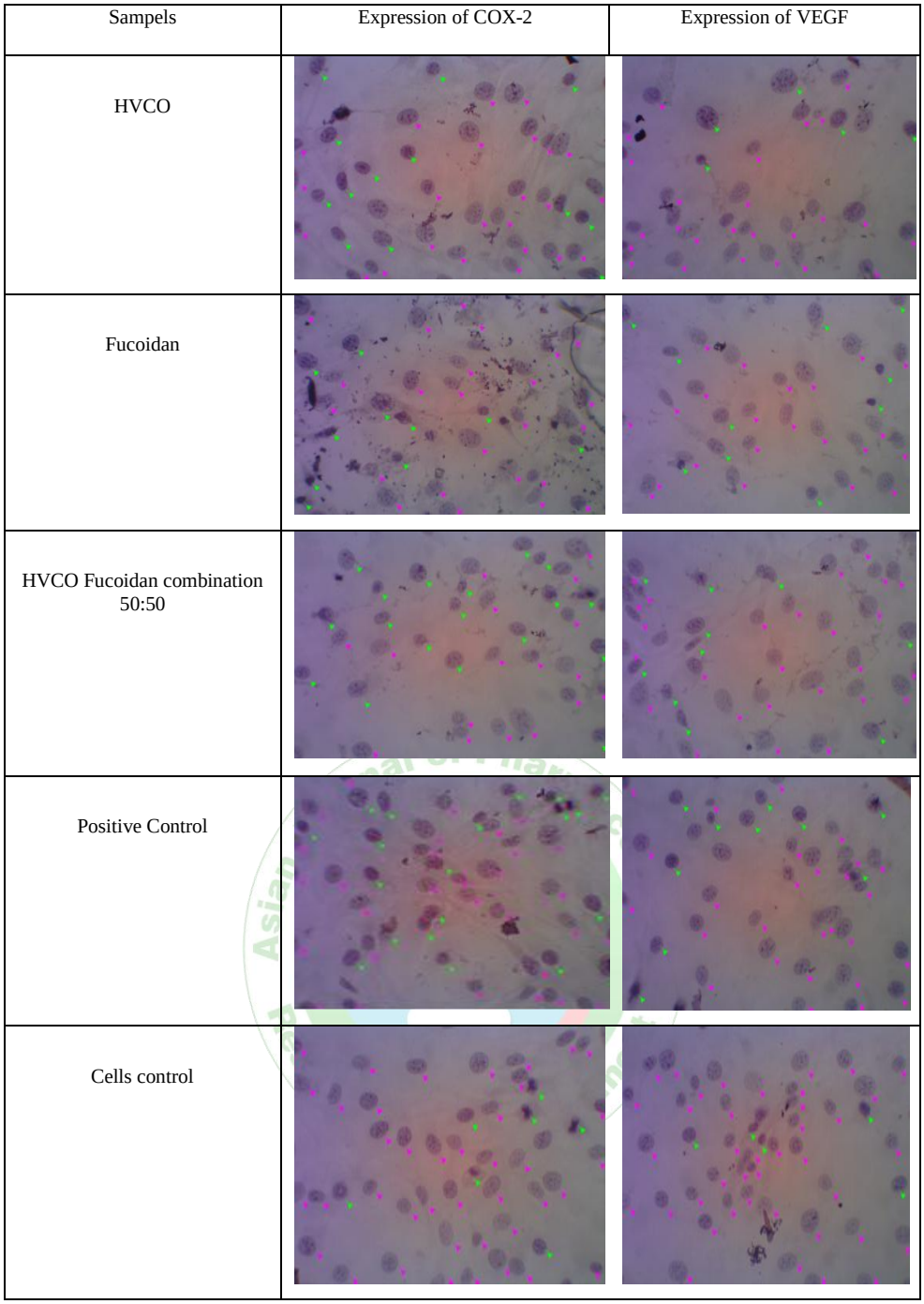

Figure 2: Observation of cells using a microscope equipped with an optilab camera (40x magnification).

: **Ekspression (+)**

: **Ekspression (-)**

Data on the percentage of COX-2 and VEGF protein expression can be seen in Table 3:

Table 3: Percentage of COX-2 and VEGF protein expression

No	Sampels	% COX-2 protein expression	% VEGF protein expression
1	Cells control	6,4816±0,29969	7,3599±0,23778
2	Aloclair®	27,1261±0,28401*	33,2574±0,36119*
3	HVCO	28,4203±0,25630*	25,4550±0,21897*
4	Fucoidan	26,8923±0,10610*	27,4068±0,24609*
5	50:50 HVCO dan Fucoidan combination	34,7558±0,09872*	30,0957±0,29865*

Values are expressed as mean± SEM using one way ANOVA; n =3 in each group
*p<0,05 : significantly different with cell control

From the percentage data above, it was found that the highest COX-2 protein expression was found in the combination test results of hydrolysis of virgin coconut oil and fucoidan 50:50, which was 34.7558%. This result was 7.8635% higher than single fucoidan (26.8923%), 6.3355% higher than the yield of single HVCO (28.4203%) and 7.6297% higher than positive control (27, 1261%). For own cell control, percent of COX-2 protein expression was 6.4816%.

Whereas the highest VEGF protein expression was found in the positive control of 33.2574%. For the samples, the combination of HVCO and fucoidan 50:50, percent protein expression amounted to 30.0957%. These results were 2.6889% higher than fucoidan(27.4068%) and 4.6407% higher than the yield of HVCO (25.4550%). For own cell control, percent of VEGF protein expression was 7.3599%.

Previous research has proven the potential of angiogenesis and wound healing from fermented virgin coconut oil, both in vivo and in vitro[5]. Park, et al. (2017) conducted a study on wound healing activity from fucoidan by in vivo. From the results of the study it can be concluded that fucoidan can accelerate incision wound healing in mice characterized by narrowing of the area of the wound and increased expression of TGF-β1, VEGFR and MMP 9[9,10].

CONCLUSIONS

The results from the proliferative activity assay showed that the best concentrations for all sampels were 31,25 µg/ml. NIH 3T3 cells migration activity assay showed that the best combination of the HVCO and fucoidan was 50:50. From COX-2 and VEGF proteins expression test results, the combination of HVCO and fucoidan had a higher percentage of expression than HVCO or fucoidan alone. From the results above can reveal that the combination of HVCO and fucoidan had better wound healing activity than HVCO or fucoidan alone with in-vitro assay.

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