

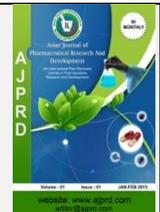


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Research Article

The Activity of Combination of Hydrolyzed Virgin Coconut Oil And Chitosan Toward Wound Healing Parameters on NIH 3T3 Cells Using In Vitro Methods

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ABSTRACT

Objectives: The objective of this study was to investigate the activity of cell proliferation of Hydrolyzed Virgin Coconut Oil (HVCO) and chitosan. HVCO and chitosan concentrations with the highest cell viability were called the best concentrations. The best concentrations of Hydrolyzed Virgin Coconut Oil (HVCO) and chitosan were combined to determine NIH 3T3 cell migration, COX-2 and VEGF protein expression.

Design In vitro cell proliferation assay was determined by MTT (Microculture Tetrazolium Technique) assay, cell proliferation activity was measured by calculating cell viability incubated 24 hours, wound closure percentage was tested by scratch wound healing method, expression of COX-2 protein and VEGF protein were measured by immunocytochemical method.

Interventions: The variable that was intervened in this study was the concentration of HVCO and chitosan.

Main Outcome Measures: The main measurements carried out in this study were the absorbance value of HVCO and chitosan which was converted into viability cell, proliferation activity, percentage of wound closure, and percentage of COX-2 and VEGF protein expression.

Results: Cell proliferation activity of HVCO and chitosan resulted the best concentration at 31.25 µg/ml, scratch wound healing assay from the combination HVCO and chitosan resulted the best migration of fibroblast cells at ratio of 50:50 with HVCO 62.5 µg/ml and chitosan 62.5µg/ml. The combination of HVCO and chitosan (50:50) increased protein expression of COX-2 and VEGF.

Conclusion: Combination of HVCO and chitosan could increase NIH 3T3 cell migration, COX-2 and VEGF protein expression. Combination of HVCO and chitosan had better wound healing activity in vitro than single use.

Keywords: *Rhizomucor miehei*, viability, proliferation, migration, expression

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INTRODUCTION

Wounds are physical damage that results in the opening or rupture of the skin which causes disruption in the anatomy and function of normal skin¹. Wound healing is an important physiological process to maintain the integrity of skin after trauma, either by accident or by intent procedure². The process of wound healing is a biological process starting from the trauma and ending with the formation of scarring. Biological process consists of hemostasis, inflammation, proliferation and remodeling. Large

numbers of cell types-including neutrophils, macrophages, lymphocytes, keratinocytes, fibroblasts, and endothelial cells are involved in this process¹⁻³. Macrophages play multiple roles in wound healing. In the early wound, macrophages release cytokines that promote the inflammatory response by recruiting and activating additional leukocytes¹. One strategy to speed up the wound healing process is to combine HVCO and chitosan. HVCO contains free fatty acids and monoglycerides especially lauric acid and active monolaurin as antibacterial and antiviral agents^{4-5,8-9,15}. Chitosan is a

biopolymer resulting from the deacetylation process of chitin which can accelerate fibroblast formation and improve the initial phase reactions associated with wound healing⁶⁻⁷.

This study was aimed to determine the activity of combination of HVCO and chitosan toward wound healing parameters on NIH 3T3 cells using in vitro methods.

MATERIALS AND METHODS

VCO (Palem Mustika®, Indonesia) was obtained from Guardian drug store, Chitosan was obtained and identified in Laboratory of Research Center of Faculty of Mathematics and Natural Science, University of Sumatera Utara, Medan, Indonesia. NIH 3T3 cells were obtained from department of parasitology, Faculty of Medicine, University of Gadjah Mada. Chemicals used were DMSO (Merck), [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) (Sigma), chromogen 3,3-diaminobenzidin (DAB) (Novo Castra), monoclonal antibody COX-2 and VEGF.

Enzymatic Hydrolysis of VCO

About 30 g of oil was weighed into 250 ml Erlenmeyer flask, and then 30 ml distilled water, 12.5 ml 0.063 M CaCl₂, 25 ml buffer Tris-HCl 1 M pH 8, and 3 ml lipase from *Rhizomucor miehei* were added into the flask. The mixture was incubated at 50°C and stirred at 200 rpm for 10 min of every 1 h incubation time (10 h). At the end of incubation time, the mixture was transferred into the separating funnel, then 50 ml n-hexane was added, and extraction was done. The mixture was allowed to stand for some time until two layers were formed. The upper layer (n-hexane fraction) was separated as the first filtrate, while the bottom layer (water fraction) was extracted again with 50 ml of n-hexane. The mixture was then allowed to stand for some time and the upper layer formed was separated as the second filtrate. The first and second filtrates were mixed, and then 250 g sodium sulfate anhydrous was added to absorb the water residue. The combined filtrate was allowed to stand for 15 min, filtered, and the n-hexane was evaporated using the water bath to obtain hydrolyzed VCO (HVCO)⁵⁻⁹.

Acid Value Determination

Five (5) g of HVCO was weighed and transferred into 250 ml Erlenmeyer flask. 25 mL of neutral ethanol 90% was added and then heated for 10 min in a water bath while being stirred, and then 3-5 drops of phenolphthalein were added into this solution. Titration was done with 0.1 N NaOH until the solution turned pink (color did not change for 15 min). The acid value and free fatty acid (FFA) percentage of HVCO were calculated using the following equation⁵⁻⁹.

$$\text{Acid value} = \frac{A \times N \times \text{MW NaOH}}{G}$$

Where,

A = NaOH solution volume (ml)

N = Normality of NaOH solution

G = Sample mass (g)

Molecular Weight of NaOH = 40 g/mol

Acid value expressed as mg NaOH/g oil was then converted to mg KOH/g oil.

Preparation of HVCO

HVCO was weighed 10 mg in the politube and dissolved in dimethylsulfoxide (DMSO) 100 µL. The sample was vortexed to dissolve completely then added with DMEM culture medium and diluted until the test solution was obtained with a concentration of 500 µg/ml; 250 µg/ml; 125 µg/ml; 62.5 µg/ml, 31.25 µg/ml and 15.625 µg/ml; all dilutions were carried out using DMEM medium containing 10% Fetal Bovine Serum (Gibco), 1% penicillin-streptomycin (Gibco), and fungizone 0.5% (Gibco)¹⁰.

Preparation of Chitosan

Chitosan was weighed 10 mg in the politube and dissolved in dimethylsulfoxide (DMSO) 100 µL. The sample was vortexed to dissolve completely then added with DMEM culture medium and diluted until the test solution was obtained with a concentration of 500 µg/ml; 250 µg/ml; 125 µg/ml; 62.5 µg/ml, 31.25 µg/ml and 15.625µg/ml; all dilutions were carried out using DMEM medium containing 10% Fetal Bovine Serum (Gibco), 1% penicillin-streptomycin (Gibco), and fungizone 0.5% (Gibco)¹⁰.

Proliferative Activity

Proliferation activity assay was carried out at the optimal concentration of HVCO and chitosan from the cell viability test. NIH 3T3 cells (1 x 10⁴ cells/well) were planted in MK DMEM, and added 100 µL test solution. After incubation for 24, 48 and 72 hours, MTT assay was performed on each well, added 100 µL of culture media and 10 µL of MTT (Sigma) 5mg/mL. Cells were re-incubated for 4-6 hours in a 5% CO₂ incubator, 37°C. The MTT reaction was stopped with a reagent stopper (10% SDS in 0.01 N HCl), wrapped plate left for one night. Absorbance was measured using Elisa Reader (Biorad) at a wavelength of 595 nm. This test is done with replication 3 times (triplo)^{10,13}.

Scratch assay

The migration test for the combination of HVCO and chitosan was carried out by making 5 series comparisons of the concentration with the highest proliferation activity in HVCO and chitosan. Comparison of HVCO and chitosan concentrations used is 100: 0; 75:25; 50: 50; 25:75; and 0: 100 from each effective concentration. Cell migration testing was carried out on NIH 3T3 cells planted in microplate 24 wells with a number of cells 5 x 10⁴ cells / well, each well 500 µl and incubated for 24 hours at 37°C. Cell cultures were washed with PBS and replaced medium with 0.5% Fetal Bovine Serum and incubated for 24 hours. Scratches were made on the bottom surface of the well using a yellow tip tip. The old media was discarded and washed in a well with 500µl PBS and taken from each group by using an inverted microscope. PBS was removed from the well by being pipetted slowly and then put the test solution 500 µL, incubated for 72 hours at 37°C. Cell conditions were observed after incubation of 0, 24, 48, and 72 hours. Microscopic images were moved in JPEG format. The initial pixel distance with an empty pixel area was

measured using Image J software. This test was done with replication 3 times (triplo)^{10,13}. The percentage was calculated by the formula:

$$Scratch = 100\% - \left\{ \frac{(\text{blank pixel area})}{(\text{initial scratch pixel})} \right\} \times 100\%$$

Immunocytochemistry

NIH 3T3 cells (1 x 10⁵ cells/well) were seeded on coverslips in 24-well plate and incubated for 24 hours. After that, the cells were treated with combination of HVCO and chitosan and then incubated for 24 hours. After incubation, the cells were washed with PBS and then fixed with cold methanol for 10 minutes then the cells were washed with PBS and blocked in hydrogen peroxide blocking solution for 10 minutes at room temperature, incubated using primary antibody COX-2 and VEGF for 1 hour, then washed twice with PBS, then incubated with secondary antibody for 10 minutes. The cells were washed with PBS, then incubated in 3,3-diaminobenzidine (DAB) solution for 10 minutes, and washed with distilled water. Afterwards, the cells were drowned with Mayer-Haematoxylin for 10 minutes, and washed with distilled water. The cover slips were taken from each well using nippers, then placed on a glass object, added with absolute ethanol, and dried. After that, added xylol and dried, then added entellan and covered with coverslip. COX-2 and VEGF expressions were observed using a light microscope with an optical camera using Raster Image Software^{10, 11, 14}. The number of protein expressions was calculated by the formula:

$$\% \text{ protein expressions} = \frac{\text{Number of cells expressed}}{\text{Total cell count}} \times 100\%$$

Statistical analysis

The data was presented as the mean ± standard error of mean (SEM). The normality of all the data was first determined by Shapiro-Wilk test. Statistical differences were determined using one-way analysis of variance (ANOVA) followed by Tukey HSD post hoc test. A value of *p* < 0.05 was considered to be significant. All analysis were performed using SPSS software version 22.0

RESULTS AND DISCUSSION

Acid Value

Enzymatic hydrolysis of VCO (virgin coconut oil) was carried out using *Rhizomucor miehei*, incubated at 50⁰C, pH 8, for 10 hours. *Rhizomucor miehei* hydrolyzed fatty acids of virgin coconut oil at the position of sn-1 and sn-3 in triglyceride molecules which produced two free fatty acids (FFA) and 2-monoglycerides^{9,15}.

Table 1: Acid value of VCO and HVCO

Sample	Acid value (mg KOH/g oil) n = 3
VCO	1.360 ± 0.315
HVCO	133.552 ± 0.610

Proliferation Activity

Proliferation is one phase in healing wounds. The proliferation phase is the phase where the fibrin matrix enters the wound area, stimulates growth factor, extracellular matrix, and angiogenesis which plays a role in the wound healing process¹⁶.

Proliferation Activity of HVCO and chitosan

HVCO and chitosan was investigated for its proliferation activity on NIH 3T3 cell lines. Viability of NIH 3T3 cells was measured using the MTT method. The absorbance data obtained from each concentration of the test material was converted to percent cell viability which was carried out three times.

Table 2: NIH 3T3 cell proliferation activity in various concentration of test material at 24 hours incubation

Treatment	Concentration (µg/ml) n = 3	Cell viability (%) (Mean ± SEM) n = 3
Cell control		100.00 ± 0.00
HVCO	500	0.47 ± 1.05*
	250	22.35 ± 3.73*
	125	132.53 ± 5.29 [#]
	62.5	148.63 ± 6.06*
	31.25	151.16 ± 5.92*
	15.625	86.22 ± 4.28 [#]
Chitosan	500	86.90 ± 6.24 [#]
	250	97.78 ± 9.90 [#]
	125	102.14 ± 7.17 [#]
	62.5	105.88 ± 8.41 [#]
	31.25	124.06 ± 4.69 [#]
	15.625	96.79 ± 1.55 [#]
VCO	500	125.28 ± 9.38 [#]
	250	129.54 ± 9.40 [#]
	125	143.83 ± 8.93 [#]
	62.5	139.70 ± 7.27 [#]
	31.25	137.95 ± 7.83 [#]
	15.625	91.43 ± 4.46 [#]

Post Hoc Tukey test, α < 0.05:

**p* < 0.05: there are significant differences with cell control

[#]*p* > 0.05: there are no significant differences with cell control

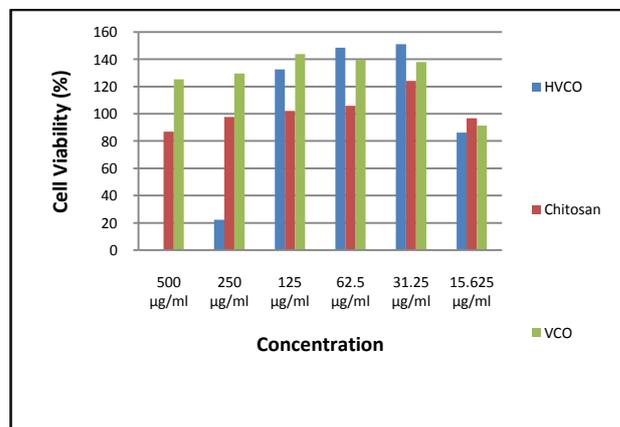


Figure 1: Percentage of viability of NIH 3T3 cell to the sample

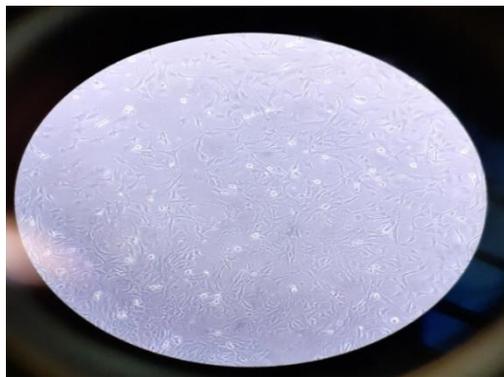


Figure 2: NIH 3T3 cell

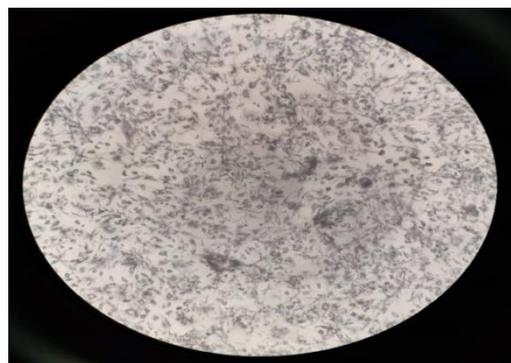


Figure 3: Formazan crystals

The highest percentage of viability of NIH 3T3 cells from HVCO found at concentrations of 31.25 µg/ml and chitosan was found at a concentration of 31.25 µg/ml. The best viability of NIH 3T3 cells from HVCO and chitosan will be continued to the cell migration test.

Lauric acid and monolaurin are antibacterial and anti-inflammatory agents that can heal wounds. Lauric acid and monolaurin can reduce the time for complete epithelialization, because lauric acid and monolaurin can increase cell proliferation and cell migration. The combination of these two components is synergistic as antibacterial and antiviral so that it can cure wound infections especially burns^{5,7}.

Chitosan provides a tissue growth matrix, activates macrophage activity and stimulates cell proliferation. Chitosan will stimulate proliferation of fibroblasts, helping to accumulate collagen regularly to stimulate the

synthesis of hyaluronic acid at the site of the wound. This speeds up the healing process and prevents scar formation^{17, 18}.

The highest proliferation activity of 3T3 NIH cells was found at concentration 31.25µg/ml for 24 hours incubation. HVCO 31.25µg/ml and chitosan 31.25 µg/ml at incubation for 24 hours had a percentage of cell viability of 151.16 ± 5.92 and 124.06 ± 4.69.

Scratch assay

Migration of 3T3 NIH cells was determined by the in vitro scratch assay method which was observed at 24, 48 and 72 hours after treatment. The HVCO and chitosan concentrations used for single were 31.25 µg/ml, while for the combination used the concentration of 62.5 µg/ml so that when combined with HVCO and chitosan the same concentration with a single concentration was obtained which was the optimal concentration.

Incubation (h)	Cell control	HVCO 31.25 µg/ml	Chitosan 31.25 µg/ml	Combination of HVCO and Chitosan (50:50)	Combination of HVCO and Chitosan (25:75)	Combination of HVCO and Chitosan (75:25)
0						
24						
48						
72						

Figure 4: Activity of wound closure of NIH 3T3 cells treated with HVCO, Chitosan and Combination of HVCO and Chitosan(50:50); (25:75); (75:25) at incubation 0; 24; 48; 72 hours by measuring the area of wound closure

Table 3: Percentage of wound closure after therapy

Treatment	Wound closure (%) (Mean ± SEM)	Wound closure (%) (Mean ± SEM)	Wound closure (%) (Mean ± SEM)
	24 jam	48 jam	72 Jam
Cell control	25.67 ± 0.11	63.70 ± 0.03	66.81 ± 0.07
HVCO 31.25 µg/ml	60.08 ± 0.06*	97.59 ± 0.02*	100.00 ± 0.00*
Chitosan 31.25 µg/ml	56.88 ± 0.05*	97.41 ± 0.03*	100.00 ± 0.00*
Combination of HVCO and Chitosan (50:50)	86.30 ± 0.00*	100.00 ± 0.00*	100.00 ± 0.00*
Combination of HVCO and Chitosan (25:75)	53.45 ± 0.04*	93.73 ± 0.00*	100.00 ± 0.00*
Combination of HVCO and Chitosan(75:25)	7.04 ± 0.07*	45.37 ± 0.02*	100.00 ± 0.00*

Post Hoc Tukey test , $\alpha < 0.05$

*p < 0.05: there are significant differences with cell control

Combination of HVCO and chitosan(50:50) has the highest percentage of wound closure (86.30% after 24 hours incubation, and 100% after 48 hours incubation). This combination is the best test material in the wound closure process. During the wound healing process, the cells on the wound edge proliferate and migrate, causing re-epithelialization of the wound surface. Lauric acid and monolaurin can increase cell proliferation and cell migration. Chitosan provides a tissue growth matrix,

activates macrophage activity and stimulates cell proliferation. Chitosan increases the activity of polymorphonuclear leukocytes, macrophages and fibroblasts which will improve the granulation and tissue regulation. Chitosan has biological activities such as wound healing, antimicrobials, hemostasis, and tissue regeneration^{5,17-19}.

Immunocytochemistry

Immunocytochemistry is a method used to detect COX-2 and VEGF protein expression in NIH 3T3 cells¹⁰.

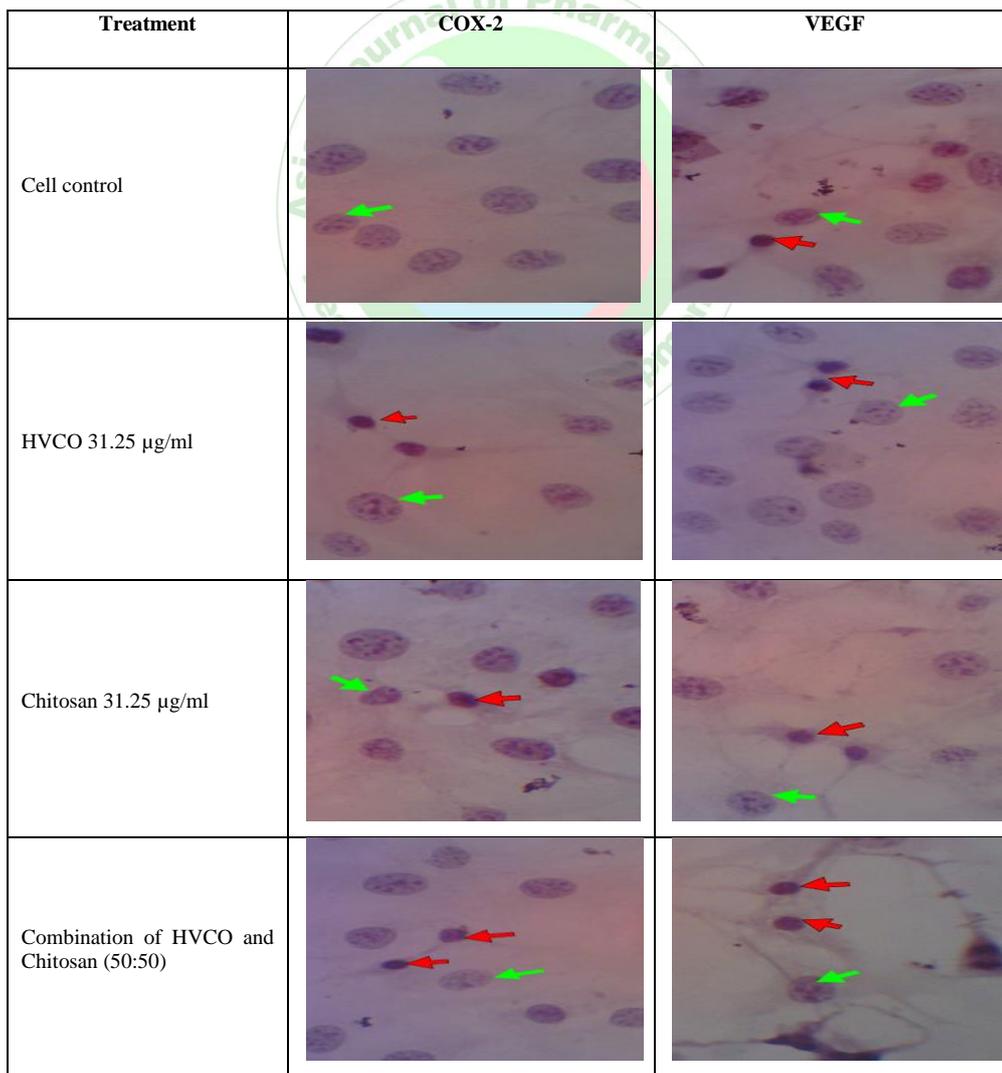


Figure 5: COX-2 and VEGF protein expression in NIH 3T3 cells after therapy

→ : Positive expression
→ : Negative expression

Table 4: Expression of COX-2 and VEGF after therapy

Treatment	Protein expression (%) (Mean ± SEM)	
	COX-2	VEGF
Cell control	16.31 ± 0,32	17.89 ± 0.19
HVCO 31.25 µg/ml	30.02 ± 0,25*	27.26 ± 0.21*
Chitosan 31.25 µg/ml	27.90 ± 0,18*	27.17 ± 0.29*
Combination of HVCO and Chitosan (50:50)	45.63 ± 0,24*	35.77 ± 0.16*

Post_Hoc Tukey test, $\alpha < 0.05$

*p < 0.05: there are significant differences with cell control

The combination of HVCO and chitosan (50:50) were stronger in stimulating COX-2 and VEGF expression and thus played a faster role in the wound healing process. COX-2 plays a key role in regulating VEGF production in fibroblasts. VEGF stimulates angiogenesis in the process of repairing wounds²⁰.

Lauric acid, monolaurine found in HVCO and chitosan increase COX-2 and VEGF expression which mediates angiogenesis and NIH 3T3 cell migration. The combination of HVCO and chitosan (50:50) was able to improve the parameters in this study namely cell proliferation activity, scratch wound healing, and COX-2 and VEGF expression. Increasing these parameters affects the factors that play a role in wound healing.

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CONCLUSION

Based on the results we obtain combination of HVCO and chitosan (50:50) could increase NIH 3T3 cell migration, COX-2 and VEGF protein expression. Combination of HVCO and chitosan had better wound healing activity in vitro than single use.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.