Original Article

เปรียบเทียบฤทธิ์ต้านไวรัสเฮอร์ปิสซิมซ์เพล็กซ์ของน้ำมันมะพร้าวสกัดเย็นและน้ำมันแร่ : การศึกษาในหลอดทดลอง

Comparison of anti-herpes simplex virus activities of virgin coconut oil and mineral oil : an *in vitro* study

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บทคัดย่อ น้ำมันมะพร้าวสกัดเย็นผลิตจากผลมะพร้าว (*Cocos nucifera* Linn) โดยการสกัดเย็นทำให้ยังคงมีฤทธิ์ทางชีววิทยา เช่น ต้านแบคทีเรียและรา ยังไม่เคยมีรายงานฤทธิ์ต้านไวรัสของน้ำมันชนิดนี้ ส่วนน้ำมันแร่เป็นน้ำมันทาผิวทั่วไป ไวรัสเฮอร์ปิสซิมเพล็กซ์มีสารพันธุกรรมเป็นดีเอ็นเอ และมีเปลือกหุ้ม อยู่ในวงศ์ *Herpesviridae* ตระกูลยา *Simplexvirus* ซึ่งประกอบด้วย HSV ชนิดที่ 1 (HSV-1) และชนิดที่ 2 (HSV-2) ทั้งสองชนิดสามารถก่อโรคติดเชื้อร้ายแรงและติดเชื้อซ้ำในคน acyclovir เป็นยาต้านไวรัสชนิดปฐมภูมิ ซึ่งมีรายงานพบ HSV สายพันธุ์ที่ดื้อยา ส่วนยาต้าน HSV อื่นๆ ยังมีราคาสูง การศึกษาเพื่อวัตถุประสงค์เพื่อตรวจฤทธิ์ต้าน HSV ของน้ำมันมะพร้าวสกัดเย็นในหลอดทดลอง โดยเปรียบเทียบกับน้ำมันแร่ วิธีการ plaque reduction assay ซึ่งถูกนำมาปรับใช้ 3 วิธี ได้แก่ inactivation, pretreatment และ posttreatment เพื่อตรวจฤทธิ์ต้าน HSV-1 (สายพันธุ์ KOS) และ HSV-2 (สายพันธุ์ Baylor 186) ผลการทดลองโดย inactivation พบว่า น้ำมันมะพร้าวสกัดเย็นความเข้มข้นร้อยละ 2 และ 2.5 (ปริมาตร/ปริมาตร) สามารถลดจำนวน plaque ของ HSV-1 และ HSV-2 ได้อย่างมีนัยสำคัญ ($p < 0.05$) เมื่อเทียบกับผลของน้ำมันแร่ ค่าความเข้มข้นที่ยับยั้งไวรัสได้ร้อยละ 50 (IC50) ของน้ำมันมะพร้าวสกัดเย็น ต่อ HSV-1 และ HSV-2 เท่ากับร้อยละ 0.51 และ 1.20 ตามลำดับ IC50 ของน้ำมันแร่ ต่อ HSV-1 และ HSV-2 เท่ากับ ร้อยละ 1.30 และ 1.80 ตามลำดับ โดย pretreatment และ posttreatment ไม่พบฤทธิ์ต้านไวรัส สรุปผลได้ว่าที่เป็นน้ำมันมะพร้าวสกัดเย็นความเข้มข้นร้อยละ 2 – 2.5 มีฤทธิ์ฆ่า HSV นอกเซลล์

คำสำคัญ: ฤทธิ์ต้านไวรัส ไวรัสเฮอร์ปิสซิมเพล็กซ์ น้ำมันแร่ น้ำมันมะพร้าวสกัดเย็น ฤทธิ์ฆ่าไวรัสนอกเซลล์

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ABSTRACT Virgin coconut oil (V) is produced from a fruit of coconut (*Cocos nucifera* Linn) by cold processing retaining biological properties, including antibacterial and antifungal activities. Antiviral property of V has not been reported. Mineral oil (M) is a common topical oil. Herpes simplex virus (HSV) is an enveloped DNA virus and a member of the family *Herpesviridae*, genus *Simplexvirus*. This genus consists of HSV type 1 (HSV-1) and HSV type 2 (HSV-2). Both HSV-1 and HSV-2 can cause serious and recurrent infections in humans. Acyclovir (ACV) is used as a primary treatment of the HSV infections. ACV-resistant HSV strains had been reported. Other anti-HSV drugs are still expensive. The objective of the study was To determine *in vitro* anti-HSV activities of V compared with those of M. For the method, plaque reduction assay (PRA) was modified into 3 methods, inactivation, pretreatment and posttreatment, for determining anti-viral effects of V and M against HSV-1 (KOS strain) and HSV-2 (Baylor 186 strain). For the results, by inactivation, 2 - 2.5% (V/V) V could reduce HSV-1 and HSV-2 plaque numbers significantly ($p < 0.05$) compared with those of M. The 50% inhibitory concentrations (ICs_{50}) of V against HSV-1 and HSV-2 were 0.51% and 1.20%, respectively. The ICs_{50} of M were 1.30% and 1.80%, respectively. By pretreatment and posttreatment**,** antiviral effect was not found. For Conclusion, 2 – 2.5% V possess virucidal effects on HSV-1 and HSV-2.

Keywords: Anti-viral activity; herpes simplex virus; mineral oil; virgin coconut oil; virucidal effect

INTRODUCTION

Virgin coconut oil (V) is widely used in Asian and Pacific regions as an edible and topical oil produced from fresh coconut milk or meat. It is a white solid fat below around 25°C (77°F), and a clear liquid oil in warmer climates.[1] Coconut (*Cocos nucifera* Linn) is in the family Arecaceae and the only viable species in the genus Cocos.[2] V is considered as the purest coconut oil and obtained by processing the mature coconut meat mechanically or naturally without heating, chemical treatment, bleaching, or deodorization, retaining many valuable components such as lauric acid, myristic acid, caprylic acid, and capric acid.[1] Lauric acid and myristic acid are resulted from V digestion whereas monolaurin is a monotriglyceride of lauric acid derived from V digestion, absorption and metabolism.[3] V has various biological properties, including antioxidant,[3,4] anti-inflammatory, analgesic and antipyretic,[5] antibacterial,[4,6-8] and antifungal activities.[4,9] Antiviral property of V has not been reported. Most antiviral activities came from lauric acid or monolaurin. Lauric acid inhibited vesicular stomatitis virus, an enveloped RNA virus.[10] Monolaurin showed *in vitro* inhibition effects on enveloped RNA and DNA viruses.[4,11]

Mineral oil (M) is a common topical oil and an ingredient in lotions, cold creams, ointments, and cosmetics. It is a lightweight inexpensive oil that is odorless and tasteless, composed mainly of alkanes and cycloalkanes from a mineral source, particularly a distillate of petroleum.[12]

Herpes simplex virus (HSV) is an enveloped spherical medium-size (155 - 240 nm) DNA virus and a member of the family *Herpesviridae*, genus *Simplexvirus*. This genus consists of HSV type 1 (HSV-1) and HSV type 2 (HSV-2). Both HSV-1 and HSV-2 can cause serious infections in humans. HSV-1 can lead to encephalitis, keratoconjunctivitis, gingivostomatitis, and skin infections whereas HSV-2 usually bring to genital infection.[13] For treatment of the infection caused by HSV, many antiviral drugs such as acyclovir (ACV), valacyclovir, famciclovir, penciclovir (PCV), and cidofovirare have been introduced. ACV is widely used as a primary treatment of this viral infection. Because of an increase in viral mutations and long term treatments, ACV-resistant HSV strains had been reported in both immunocompromised and immunocompetent patients. The prevalence of ACV-resistant HSV was higher in severely immunocompromised patients than in immunocompetent patients. PCV-resistant HSV was obtained from immunocompetent patients.[14-17] Many anti-HSV drugs are still expensive. Edible and topical oil with low cost of production such V attracted our attention to investigate ant-HSV effects of V. Therefore, this study aimed to determine *in vitro* antiviral activities of V against HSV-1 and HSV-2 compared with those of M by using plaque reduction assay.

MATERIALS AND METHODS

1.Preparation of tested samples: V and M used in this study were produced by Chemipan Laboratories Co., Ltd. (Thailand); and Johnson & Johnson (Thailand), respectively. Polyethylene glycol (PEG) 400 (Chemipan Laboratories, Thailand) was used as an emulsifying agent to make oil in water (O/W) emulsion in a ratio (v/v) 1:1 (PEG 400:oil). The certificate of Analysis (COA) (from the producer) of V was reported that it contained 0.047% lauric acid by AOAC method. Briefly, V or M was mixed thoroughly with PEG 400. The mixture were then added to minimum essential medium (MEM, Invitrogen®, USA) and mixed thoroughly to make an emulsion. The final concentrations of tested oil after mixing with the virus (tested sample) or MEM (cell control) in a ratio (v/v) 1:1 were 1 - 4% (v/v). MEM was mixed thoroughly with 1 - 4% (v/v) PEG 400 to make an emulsion and then

mixed with the virus in a ratio (v/v) 1:1 to be a negative control of each test sample.

2. Preparation of tested viruses and cell culture: HSV-1 (KOS strain), HSV-2 (Baylor 186 strain) and Vero cells (kidney cells of an African green monkey) obtained from the National Institute of Health, Thailand were used as tested viruses and cell culture. Vero cells are widely used for HSV-1 and HSV-2 infections. The cells were propagated confluently in a growth medium (GM) (MEM supplemented with 10% heated fetal bovine serum, Invitrogen®, South America) on the surface of a 25 cm^2-tissue culture flask in an incubator at 37°C and 5% CO_2 (Forma Scientific, USA) for 18 - 24 hr. After the GM was taken off, 2 mL of MEM containing the viruses were inoculated on confluent Vero cells with a multiplicity of infection (MOI) ≤ 0.01. After viral adsorption for 1 hr and then unadsorbed viruses were removed, 4 mL of MEM were added and the culture cells were reincubated for 2 - 3 days or until 90% cytopathic effect (CPE) were observed under an inverted microscope. The viruses were then harvested and collected as the stock viruses and kept at - 20°C until used. Quantification of the stock viruses were determined by a plaque assay. All the tested samples, cells, viruses were prepared and tested in a biosafety cabinet class II (Esco®, Singapore)

3. Plaque assay: A monolayer of Vero cells prepared on each well of a 24-well (for HSV-1) or 12-well (for HSV-2) tissue culture plate at 37°C and 5% CO_2 for 18 - 24 hr. After removing the GM, the stock viral solution was 5-fold diluted with MEM and then 0.25 (for HSV-1) or 0.5 (for HSV-2) mL of each dilution was added on Vero cells in each well (3 wells/dilution). The culture plate was reincubated for 1 hr. An overlayer medium (MEM containing 1.6% methylcellulose, 0.25 mL for HSV-1 or 0.5 mL for HSV-2) was added on the cells in each well and the plate was then reincubated for 2 - 3 days or until visible plaques were seen. Fixing solution (38% formalin:normal saline, 1:2) was added to and mixed with the overlayer medium in each well (0.5 mL/well for 24-well plate and 1 mL/well for 12-well plate). After fixing the infected cells at room temperature for 1 hr, the mixture solutions were removed and the cells were stained with 1% methylene blue with 10% methanol solution at room temperature for 1 hr. The extra methylene blue solution was washed with tapped water and plaques were counted in each well under an inverted microscope. The viral stock concentration was calculated as plaque forming unit (PFU)/mL.[18]

4. Plaque reduction assay (PRA): PRA was modified into 3 methods, inactivation, pretreatment and posttreatment, for determining anti-HSV activities of all samples as below.[19]

4.1 Inactivation: Appropriate amount of viruses (500 - 700 PFU/mL) was mixed with each concentration of the test sample or a control (MEM) in a ratio (v/v) 1:1 for 30 min at room temperature. The mixture were then added on each well (3 wells/concentration) containing Vero cell monolayer of a 24-well (for HSV-1, 250 µL/well) or 6-well (for HSV-2, 1.0 mL) tissue culture plate immediately after removing the GM. The culture plate was incubated at 37 °C and 5% CO_2 for 1 hr. The overlayed medium was added on the cells in each well (0.25 mL for HSV-1 or 1.0 mL for HSV-2) and the plate was then reincubated for 2 - 3 days or until visible plaques were seen. The infected cells were fixed and stained. The plaques in each test concentration were counted and calculated for viral titers. Dose-response curves were used to determined 50% inhibitory concentrations (ICs_{50}) of V and M.

4.2 Pretreatment: Monolayer Vero cells of each well of 24-well (for HSV-1) or 6-well (for HSV-2) tissue culture plate after removing the GM was pretreated with each concentration of tested samples or control, triplicately in the CO_2 incubator for 1 hr. Appropriate amount of viruses were adsorbed on each pretreated cells of the wells in the CO_2 incubator for 1 hr. The overlay medium was added to the wells. The plaques were enumerated and the ICs_{50} of the samples were calculated after 2 - 3 days.

4.3 Posttreatment: Appropriate amount of viruses were adsorbed on monolayer Vero cells of each well of 24-well (for HSV-1) or 6-well (for HSV-2) tissue culture plate immediately after removing the GM for in the CO_2 incubator 1 hr. The tested samples were added onto the infected cells and reincubated for 1 hr. The overlay medium was added to each well of the infected cultures. The ICs_{50} of the samples were calculated as previously mentioned.

5. Statistical analysis: All experiments were performed in triplicate, and three independent experiments were conducted. Data were analyzed using SPSS 29 (IBM, Armonk, New York, USA) (independent-samples Kruskal-Wallis test and Mann-Whitney test) and reported as means ± SD. p-value < 0.05 was considered to indicate a statistically significant difference.

RESULTS

1. Cytotoxicity to Vero cells.

In this study, we found that Vero cells could not survive in the MEM containing 3 - 4% V or 3 - 4% M. Cytotoxic effects of the tested oil with more than or equal to 3% on the culture cells were found. Consequently, we continued to determine antiviral effect of 1.0 - 2.5% V and M. We also discovered that polysorbate 20 was not a suitable emulsifier for our study because it alone inhibited the viral titer and destroyed Vero cells. We found that polysorbate 20 less than or equal to 0.6% was safe for HSV and Vero cells but it was too low to emulsify V or M. An emulsifier helped an oil to dissolve in water and the

oil could react with the viruses. We chose PEG 400 as an emulsifying agent because it was nonionic and the used concentrations (1.5 - 2.5%) did not inhibit the viral titer or not destroy Vero cells.

2. Antiviral effects

By inactivation method in the PRA, the results showed that 2 - 2.5% V could reduce HSV-1 plaque numbers significantly ($p < 0.05$) compared with M (the same concentrations). In addition, 2 - 2.5% M could decrease HSV-1 plaque numbers significantly ($p < 0.05$) compared with the negative controls (MEM containing 2 - 2.5% PEG 400). The inhibition effects of V and M on HSV-1 were dose-dependent (Figure 1). The 50% inhibitory concentrations (ICs_{50}) of V and M against HSV-1 were 0.51% and 1.30%, respectively (Table 1).

Similarly, the results demonstrated that 2 - 2.5% V could lower HSV-2 titers significantly ($p < 0.05$) compared with M (the same concentrations). The inhibition effects on HSV-2 of V and M were also dose-dependent (Figure 2). The ICs_{50} of V and M were 1.20% and 1.80%, respectively (Table 1).

By pretreatment and posttreatment methods in the PRA, the results showed that V as well as M could not reduce HSV-1 and HSV-2 plaque numbers.

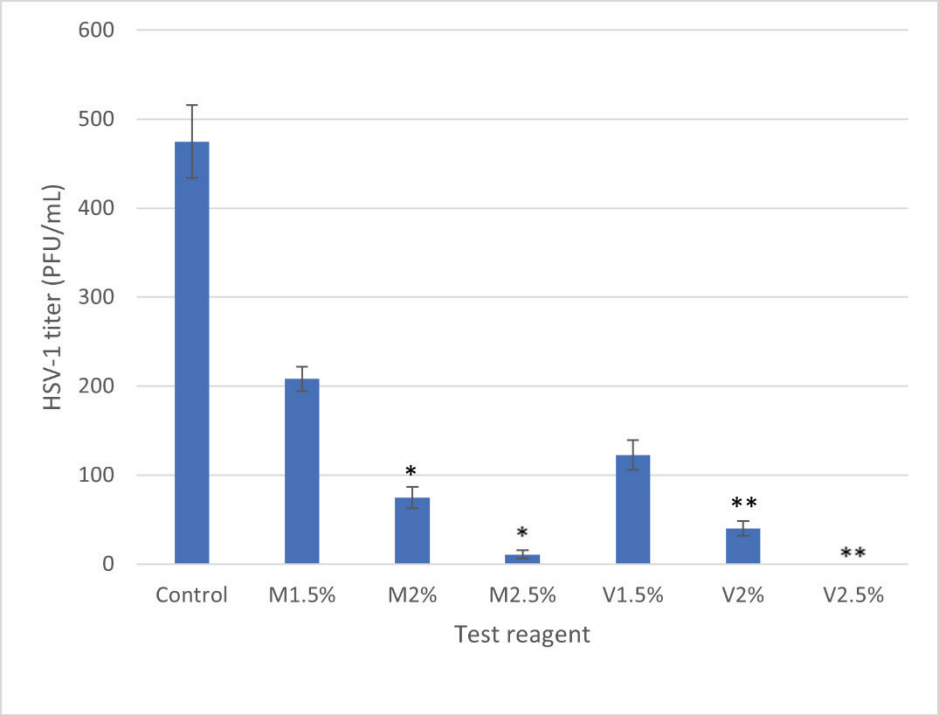

Figure 1 Effects of virgin coconut oil (V) and mineral oil (M) on herpes simplex virus type 1 (HSV-1)(KOS strain) determined by plaque reduction assay (inactivation). Results were represented as mean (n = 3) ± SD. * represented as different from the negative controls (minimum essential medium containing PEG 400) and ** represented as different from M significantly ($p < 0.05$)

Table 1 Comparison the 50% inhibitory concentration (IC_{50}) of virgin coconut oil and mineral oil against herpes simplex virus type 1 (HSV-1) (KOS strain) and herpes simplex virus and type 2 (HSV-2)(Baylor 186 strain) determined by plaque reduction assay (inactivation)(n = 3). Minimum essential medium containing PEG 400 as negative controls.

Test reagent	IC_{50} (mean ± SD)(%)	
	HSV-1	HSV-2
Mineral oil	1.30 ± 0.05	1.80 ± 0.04
Virgin coconut oil	0.51 ± 0.22	1.20 ± 0.09

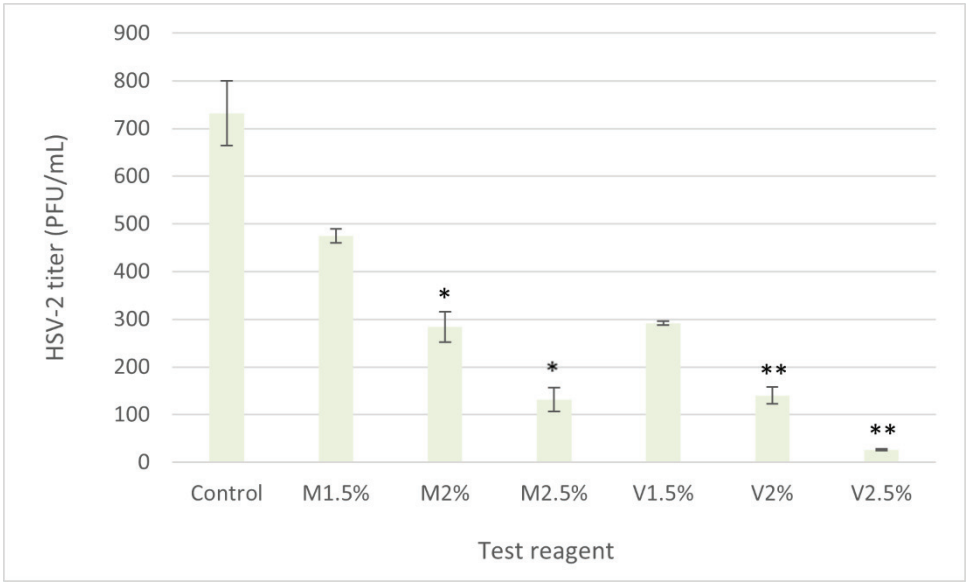

Figure 2 Effects of virgin coconut oil (V) and mineral oil (M) on herpes simplex virus type 2 (HSV-2)(Baylor 186 strain) determined by plaque reduction assay (inactivation). Results were represented as mean (n = 3) ± SD. * represented as different from the negative controls (minimum essential medium containing PEG 400) and ** represented as different from M significantly ($p < 0.05$).

DISCUSSION

In this study, we compared V with M because M is a common topical oil. Agero and Verallo-Rowell[20] determined the effectiveness and safety of V compared with M as a therapeutic moisturizer for mild to moderate xerosis by a randomized double-blind controlled clinical trial. Subjective grading of xerosis by the investigators showed a general trend toward better (though not statistically evident) improvement with V than with M. They concluded that V was as effective and safe as mineral oil when used as a moisturizer.

Antibacterial and barrier repair properties of V and M were reported by Ramos[21] in children with mild to moderate atopic dermatitis. By using SCORAD (SCORing for Atopic Dermatitis) and bacterial culture, a randomized controlled doubleblind trial was conducted in two tertiary hospitals. The SCORAD and level of erythema were significantly decreased throughout the 4-week duration for both treatment groups, but much lower in the V group. The antibacterial property of V group was better than that of M group since there was no growth of bacteria after the 4-week treatment.

V and M were compared with the control using Kruskal-Wallis test. The nonparametric Kruskal-Wallis test is preferable to a one-way analysis of variance with at least three groups of independent samples, the differences between these sets of data estimated.[22] V was compared with M using the Mann-Whitney test. The Mann-Whitney U test is the nonparametric equivalent to the two samples t-test when the dependent variable is a continuous variable measured for all observations in two groups.[23]

Plaque formations were significantly reduced, when HSVs were incubated with certain amount of V or M prior to adsorption indicating that V and M had virucidal effects or direct inactivation on HSV-1 and HSV-2. Interestingly, V exhibited virucidal effects greater than those of M significantly. The same results were found when star anise oil was examined for antiviral activity against HSV-1 using PRA. Anti-HSV-1 activity of star anise oil was direct inactivation of free virus particles.[24] The antiviral effects of peppermint oil against HSV-1 and HSV-2 were tested *in vitro* on RC-37 cells using PRA. This oil affected the viruses before adsorption, but not after penetration into the host cells.[25]

In order to determine the mode of antiviral action of V and M, either Vero cells were pretreated before viral infection or viruses were incubated with the tested oils before infection or tested oils were added after viral adsorption on the host cells. Pretreatment of Vero cells with V or M had no effect on the production of infectious HSV and plaque formation. So, V and M could not prevent penetration of the viruses to their susceptible cells . To observe the intracellular anti-HSV activities of the tested samples, the posttreatment experiment was carried out. V and M had no intracellular effect on HSV infection. Future study about a more emulsifier may approve the intracellular effect since this effect is important for treatment of an infectious disease.

V is composed of valuable components such as lauric acid (C12, a major component), myristic acid (C14), caprylic acid (C8), capric acid (C10). However, coconut oil contained only trace amounts of free fatty acids (about 0.03% by mass).[1] Caprylic acid and capric acid are short chain fatty acid (SCFA). Lauric acid and myristic acid are medium chain fatty acid (MCFA).[3] V, primarily a MCFA is not packaged into chylomicrons for circulation through lymph vessels like long-chain fatty acids (LCFA), and instead is broken down quickly upon consumption instead of being stored in adipose tissues. MCFA is also easily soluble and digestible by salivary and pancreatic lipases when compared to LCFA.[4] Many studies suggested lauric acid had better antimicrobial properties amongst all fatty acids, including monolaurin, a secondary metabolite. Vesicular stomatitis virus (VSV) is an enveloped RNA virus that infects cattle, horses, and pigs. In the presence of lauric acid (C12) (purchased from Sigma)(60 - 100 µg/ml), the production of infectious VSV was inhibited reversibly in a dose-dependent manner. After removal of C12 the antiviral effect disappeared. In addition, the chain length of the monocarboxylic acids proved to be crucial, as those with shorter or longer chains were less effective or had no antiviral activity. C12 prevented the binding of the viral M protein to the host cell membrane, resulting in inhibition of virus release. Thus, lauric acid had an intracellular effect on VSV.[10] In our study, 2 – 2.5% V containing few lauric acid (about 2.35 - 2.94 µg/ml) showed anti-HSV effects. Physical property of the oil may help virucidal effect. However, the comparison was difficult because of different methods and evaluations. We used PRA and evaluated V as IC50 values. Hornung et al.[10] used viral titer assay and evaluated lauric acid as 10-fold viral reduction.

Monolaurin (obtained from Med Chem Laboratories, Monroe, MI, USA) with tert-butyl hydroxyanisole (BHA) (used for prevent oil from oxidative deterioration) showed *in vitro* virucidal effects on human enveloped RNA (influenza virus, pneumovirus, paramyxovirus, rubeola virus) and DNA viruses (HSV-1, HSV-2, cytomegalovirus). When the 1% concentration was added to the reaction mixture, after 1 hr (but not 30 min) at 23°C, all viruses were reduced in infectivity by more than 99.9%. Electron micrograph of the treated viruses showed that loss of viral infectivity was associated with disintegration of the virus envelope. Destruction of lipids and phospholipids in the viral envelope is the key factor in the virucidal activity of monolaurin.[11] Our results showed that V had virucidal effect after inactivation at least for 30 min (but not 15 min). The virucidal effects of V and M on enveloped DNA viruses like HSV-1 and HSV-2 may come from a physical effect.

Antiviral property of V has not been reported. Moreover, antiviral effects of lauric acid[10] or monolaurin[11] reported previously were produced from commercial companies not extracted from V.

CONCLUSION

The results of this study showed that 2 - 2.5% V possess in vitro virucidal effects against HSV-1 and HSV-2 better than those of M.

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CONFLICT OF INTEREST

The authors declare that we have no financial interests or personal relationships that could be influent the results in this study.

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