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Research Article

IN VITRO BIOACTIVITIES OF CLOVE BUDS OIL (*Eugenia caryophyllata*) AND ITS EFFECT ON DERMAL FIBROBLAST

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ABSTRACT

The aim of this study was to investigate the antioxidant and antityrosinase activities of clove oil and its components, the correlation between their antioxidant and antityrosinase activity and to examine the effect of clove bud oil towards dermal fibroblasts. The results show that the major component presented in clove oil is eugenol (99.16%). Free radical scavenging capacity of clove oil using the DPPH method showed significant correlation with lipid peroxidation inhibition using TBARs method (r=0.804) but less correlation with tyrosinase inhibition (r=0.576). Linear regression analysis revealed that free radical scavenging capacity was more correlated to tyrosinase inhibition than lipid peroxidation inhibition. In addition, clove buds oil exhibited cytotoxicity at IC_{50} of 0.162 µl/ml and significantly increased collagen synthesis at as low as 0.0156 µl/ml. In conclusion, clove buds oil might possess whitening effect and anti-aging effect. However, due to its cytotoxicity, clove buds oil should be used in very low concentrations with care.

Keywords: Clove oil, Antioxidant index, Tyrosinase inhibition, Collagen synthesis, Cytotoxicity

INTRODUCTION

Skin aging occurs with time and is caused by environmental factors. In aged skin, slower turnover causes thinning of the epidermis that gives aged skin a translucent appearance, resulting in dyschromic skin changes. In the elderly dermis, the decrease of fibroblast cells affects decrease of collagens and elastin fibers synthesis, resulting in skin wrinkling and loss of elasticity[1]. In addition, photoaging skin damage by reactive oxygen species (ROS) may reduce the strength of skin cell walls, as well as degrading collagen and elastic fibers, resulting in loss of skin humidity and elasticity leading to skin wrinkling. Application of topical antioxidant cosmetic products may prevent intrinsic and extrinsic skin aging[1-2]. However, sunlight can induce skin tanning by ROS which may activate tyrosinase enzyme to catalyze the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones. Then the *o*-quinones can change L-tyrosine to L-DOPA and L-DOPA to L-dopaquinone, resulting in melanin pigments[3-4]. As a result, inhibition of tyrosinase enzyme by antioxidants may reduce melanogenesis.

Essential oils are commonly used in traditional medicine and widely used in cosmetics. Many studies have demonstrated that essential oils have anti-inflammatory activity, antibacterial activity and antioxidant activity. Because inflammation is commonly related with oxidative damage, substances which can inhibit inflammation may also be antioxidants via free radical scavenging and lipid peroxidation inhibition [5-9]. Clove (*Eugenia caryophyllata*) is the aromatic dried flower buds of a tree in the family Myrtaceae. The essential oil of clove is used as anti-mutagenic [10], anti-inflammatory and antioxidant activities [11-13]. The aims of this study were to investigate *in vitro* bioactivities, the correlation of antioxidant activity and tyrosinase inhibition of clove oil and its components as well as the effect on dermal fibroblasts.

MATERIAL AND METHODS

Materials

Clove oil was purchased from Thai China Flavours & Fragrances Industry Co. (Thailand), butylated hydroxyl toluene (BHT) and alpha-tocopherol acetate (vitamin E acetate), Eugenol and trans-caryophyllene, were purchased from Sigma Chemicals (USA). Thiobarbituric acid (TBA), Chloramine T reagent, Ehrlich's reagent, Trichloroacetic acid (TCA) Trans-caryophyllene, 2, 2-azole-(2-aminopropane)-dihydrochloride (ABAP) were obtained from Sigma Aldrich (Switzerland), calcium chloride ($CaCl_2$), L-Tyrosine, Mushroom tyrosinase enzyme, 2, 2-diphenyl-l-1-picrylhydrazil (DPPH), tris-base buffer, sulforhodamine B (SRB) were purchased from Fluka, Switzerland. All culture media and culture supplements were purchase from Gibthai (Thailand).

Identification and quantification of clove oil components

GC/MS analysis of the essential oil sample (1%v/v in Dicholoromethane; DCM) was carried out on an Agilent Technologies (China), Model CN 10402086, equipped with a column DB-5ms (0.25mm x 30m x 0.25µm ID). The carrier gas used was helium at a flow rate 1ml/min. The column temperature started with 70°C for 5 min, then programmed at 3°C /min to 120°C for 2 min and at 5°C/min to 270°C. Sample (1µl) was injected neat with 1:100 split ratios. Mass spectra were recorded in scan mode 35-550m/z with scan rate 1388.2amu/s and the ion source temperature was 230°C. The components were identified by their linear retention indices and compared with their mass spectra with the NIST MS Search Library and the reference compounds.

To determine the quantity of clove oil and reference compounds, ethanolic solution of clove oil (0.2%v/v) or the synthetic reference compounds (0.004-0.2 %v/v) in DCM were prepared and analyzed using a gas chromatograph connected to a flame ionization detector (FID) (GC 1850, Agilent). A HP-5 (30 m x 0.32 mm id. x 0.25 µm film thickness) capillary column was used. The injector temperature was 250°C, The oven temperature was started at 100°C and held for 1 min. Temperature programming was increased from 100°C to 220°C at 10°C/min and held for 1 min. The carrier gas was nitrogen at a flow rate of 2 ml/min while the split ratio was 1:10. The effluent was detected by FID at 280°C. A calibration curve was constructed by plotting between concentrations of either reference compounds or clove oil and their peak area.

Free radical-scavenging activity: 2,2-diphenyl-1-picrylhydrazyl (DPPH) test

The free radical-scavenging activity of essential oil was measured according to the method modified from Lertsatitthanakorn et al.[6]. Briefly, an equal volume (50µl) of a sample solutions which were the ethanolic solution of clove oil (20 µl/ml), eugenol (20 µl/ml) and β-caryophyllene (400µl/ml) was mixed with 0.156 mg/ml DPPH ethanolic solution. 50µl of the solutions were two fold serially diluted in absolute ethanol and mixed well. Then, a 50µl DPPH was added to each well. The reaction mixture was mixed for 5min and incubated in the dark for 25min, and then the OD measured at 490 nm on a microplate reader. α-tocopheryl acetate (vitamin E acetate) and butylated hydroxyl toluene (BHT) were used as the positive controls while ethanol alone served as a negative control.

$$\text{Antioxidant Index (\%)} = [1 - (A_{samp} - A_{blk}) / A_{cont}]*100$$

Determination of antioxidant activity by inhibition of lipid peroxidation (TBARs)

The thiobarbituric acid reactive species (TBARs) assay was used to measure the potential antioxidant capacity of the compounds by modifying the method from Ruberto and Baratta [14], using egg yolk homogenates as lipid rich media. The stock solutions of tested samples were prepared in absolute ethanol with the following concentrations; 10 mg/ml of clove oil or eugenol, 100 mg/ml of β-caryophyllene, 0.10 mg/ml of BHT and 100 mg/ml of vitamin E acetate. Briefly, 50μl of 10% (w/v) egg yolk in 1.15% w/v KCl, prepared immediately before use, was added into a 1.5-ml Eppendrof. 10μl of the sample stock solution was added and 40μl of distilled water was added to make a final volume of 100μl. 5 μl of ABAP solution (0.07M) in water was added to induce lipid peroxidation, and then the reaction was incubated at room temperature for 30 min. Then, 150μl of 20% acetic acid (pH 3.5) and 150 μl 0.8% (w/v) thiobarbituric acid (TBA) in 1.1% (w/v) sodium lauryl sulphate (SLS) solution was added and the resulting mixture was mixed well before heating at 95°C for 60 min. After the mixture had cooled down, 500 μl of butan-1-ol was added and centrifuged at 1200xg for 10min. After that, 200 μl of the organic upper layer of each sample was removed and added into the 96-well micro plate for measuring the absorbance at 550 nm. Vitamin E acetate and BHT were used as the positive controls, absolute ethanol was used as a negative control, and distilled water was used as a blank.

$$\text{Inhibition of lipid peroxidation (\%)} = [1 - (A_{samp} - A_{blk}) / A_{cont}]*100$$

Determination of mushroom tyrosinase inhibition activity

The tyrosinase inhibition activity of clove oil was measured according to the method modified from Marongiu et al.[15]. L-tyrosine was used as the substrate in this experiment. Firstly, 30 μl of clove oil and its component concentrations were dissolved in 10% v/v of DMSO. 30μl of the clove oil solutions were two fold serially diluted in 0.1M phosphate buffer (pH 6.8). Then, 120μl of the phosphate buffer was added to make the final volume 150μl and it was mixed well. After that, 50μl of L-tyrosine solution (0.30mg/ml of L-tyrosine in phosphate buffer pH 6.8) was added to each well. Finally, 50μl of mushroom tyrosinase enzyme solution (0.04mg/ml of mushroom tyrosinase enzyme in phosphate buffer solution) were added. The resulting mixture was then incubated at 30°C for 10 min and left to stand at room temperature for 15min prior to measuring the absorbance at 490nm. Kojic acid solution was used as a positive control, and phosphate buffer solution was as a negative control.

$$\text{Inhibition of tyrosinase enzyme activity (\%)} = [1 - (A_{samp} - A_{blk}) / A_{cont}]*100$$

Where, A_{samp}, A_{blk} and A_{cont} are the OD of the sample, blank and negative control, respectively.

Effect of clove oil and its components on dermal fibroblasts

Human skin fibroblast culture

Human skin fibroblast cell lines provided by Assistant Professor Dr. Wilairat Leeanansaksiri, The School of Biology, Institute of Science, Suranaree University of Technology, was used in this study. To prepare the cell suspension, the dermal fibroblasts were cultured in high glucose-DMEM supplemented with 10% fetal bovine serum, 1%w/v penicillin-streptomycin and maintained at 37 °C in 5% CO_2/air until sub-confluences. The cell monolayer was trypsinised with 0.25% trypsin/EDTA solution at 37 °C for 20 min and washed with the culture media before use.

Cytotoxicity test using sulforhodamine B (SRB) colorimetric assay

Cytotoxicity of clove oil and its major components were examined by the SRB assay [16]. 190μl the cell suspension was seeded at density of 10^4cells/well. After 24h of seeding cell suspension, 9 wells of cell monolayer were fixed by 100μl 10% (w/v) trichloroacetic acid (TCA) at 4°C for 1h after discarding the old medium, then clove oil and eugenol concentrations were serially diluted from 10μl/ml to 0.15625μl/ml, and β-caryophyllene concentration were from 2μl/ml to 0.03125μl/ml in absolute alcohol. 10μl of each concentration was exposed to a 96-well plate containing confluent cell monolayers in day1 (after 24 h of seeding cell suspension). 10μl ethanol and 10μl media without serum were used as controls. After 72 h, the treated cell monolayers were fixed by 100μl of 10% (w/v) TCA. After the 96-well plates were dried, 100μl of 0.057% (w/v) SRB solution was added to each well and the plates were shaken on a titer shaker for 5 min to dissolve the protein-bound dye and the absorbance measured at 490nm using a microplate reader.

$$\text{\% cell killed} = 100 - (\text{mean } OD_{samp} / \text{mean } OD_{day0})*100$$

Quantification of collagen synthesis

A 10 μl of the stock sample solutions at various concentrations was added into 190 μl of the cell suspension to make a final density of 10^4cells/well in a 48-well plate. The plate was kept at 37 °C in 5% CO_2/air for 3 days. Then, the cell layer was trypsinised at 37 °C for 20 min and collected as cell lysate solution. Collagen assay was performed according to the study of Lin and Kuan [17] with some modification. Briefly, coated 48-well plates were prepared accordingly; 200 μl of 1%w/v sodium acetate in ethanolic solution were added into each well and thoroughly coated the well before air-dried. An equal amount of the cell lysate solution was mixed with 4N NaOH solution and hydrolyzed at 121 °C for 40 min. To determination of collagen content, twenty microliters of the hydrolysed solutions were mixed with 30 μl of 2 N NaOH solutions in the coated 48-well plate. The mixture solution was then mixed with 450 μl of buffered chloramine T reagent for 25 min at room temperature. Then, 500 μl of Ehrlich's reagent was added and incubated at 65 °C for 40 min. The incubated plates were gradually cooled down at room temperature for 10 min then at 4 °C for 15 min and kept at room temperature for 20 min before measuring the absorbance at 550 nm using microtiter plate reader (Biorad, USA). 4-Hydroxyproline (4-hyp) solutions (0-100μg/ml) were prepared in the same manner as test samples and used as a collagen standard curve. The percentages of collagen synthesis on day 3 were calculated in comparison with the collagen content of fibroblasts at day 0.

Statistical Analysis

All experiments were replicated three times and all measurements were performed in triplicate. The differences of bioactivities among clove oil, its major components and reference standards were analyzed using one-way analysis of variance (ANOVA). Differences were considered to be significant at <0.05. A linear regression analysis was carried out to determine the correlation between antioxidant index and tyrosinase inhibition.

RESULTS AND DISCUSSION

GC/MS chromatograms showed that the major component in clove oil was eugenol (99.16%), followed by β-caryophyllene (0.30%) and others (0.54%) (Table1). In this study, clove buds oil was practically composed of a large amount of eugenol followed by a small amount of β-caryophyllene. It was found that the quantity of eugenol was greater than that found in the study of Dorman et al. [18].

Table 1: The main components of clove oil

Chemical formula	Component	RI	Percent Area	Mode of Identification*
$C_{10}H_{12}O_2$	eugenol	1350	99.16	a, b, c
$C_{15}H_{24}$	β- caryophyllene	1416	0.30	a, b, c

*a = mass spectra; b = RI; c = authentic compounds

Table 2: Bioactivities of clove oil and its major components compared with reference standards

Substance	IC_{50} (µg/ml)		
	DPPH	TBARs	Tyrosinase Inhibition
Clove oil	134.2±4.9	942.0±49.6[a]	9.6±1.5
Eugenol	145.2±44.3	382.0±35.4[a]	8.2±4.2
β-caryophyllene	10449.0±1067.3[a]	4917.8±60.8[b]	17.0±0.2[a]
BHT	84.4±25.6[ab]	63.1±15.6[ab]	ND
Vitamin E acetate	41332.4±7984.7[a]	3651.5±395.4[b]	ND
Kojic acid	ND	ND	0.007±0.001[ab]

[a,b]$p< 0.05$

ND: not determined

The concentrations of samples resulting in a 50% inhibition of DPPH free radical, TBARs and tyrosinase inhibition, IC_{50}, are shown in Table 2. The results demonstrate that clove oil and eugenol possess free-radical scavenging ability which is more pronounced than their lipid peroxidation inhibition. Although the free-radical scavenging activity of clove oil is not significantly different from eugenol, the inhibition of lipid peroxidation of eugenol is more significantly potent than clove oil. However, both clove oil and eugenol exhibit less antioxidant activity than BHT. In addition, β-caryophyllene exhibited weak antioxidant activity. In comparison with vitamin E acetate, β-caryophyllene possessed more lipid peroxidation inhibition than its free-radical scavenging activity. In the DPPH test, free radical scavenging antioxidants act by donating hydrogen atoms to DPPH radicals. The stable radicals obtained from antioxidants then enable the stopping of the oxidation chain reaction. The reaction mechanism between the antioxidant and DPPH radicals depends on the structural conformation of the antioxidant[5,19-20]. Eugenol is a natural phenolic compound which more easily donates hydrogen than β-caryophyllene which is a sesquiterpene hydrocarbon, and vitamin E acetate, which is an ester compound, requires esterase enzyme to convert into vitamin E which is an active antioxidant. As a result, eugenol possesses more potent free radical scavenging capacity than β-caryophyllene and vitamin E acetate. However, antiradical action of eugenol was less effective than that of BHT. The results can be explained as proposed by Bondet et al. [19] that eugenol reacts with DPPH radicals in a dimerization mechanism with a stoichiometry of 1.9 whereas BHT reacts with DPPH radicals in three different pathways including complexation, hydrogen atom delocalization and dimerization with a stoichiometry of 2.8.

In the TBARs assay, antioxidants react with peroxyl radicals to inhibit the propagation cycle of lipid peroxidation. The ability of antioxidants on inhibition of lipid peroxidation reaction in lipid-rich substrate was examined by measuring the formation of malonaldehyde which is the secondary oxidative product of the lipid substrate [5,18]. In this study, inhibition of lipid peroxidation of eugenol was much greater than that of clove oil. Although clove oil is composed of eugenol as a major component, the lipid peroxidation inhibition of the component was concentration dependent. Besides, clove oil also contains a small amount of β-caryophyllene which possesses lesser inhibition capacity. Moreover, the correlation between DPPH and lipid peroxidation of eugenol was 0.639 while that of clove and β-caryophyllene was 0.804 and 0.922, respectively (Table 3). As a result, antioxidant activity of eugenol and clove oil in lipid-rich substrate was significantly different. Furthermore, free radical scavenging capacity of eugenol and clove oil was stronger than inhibition of lipid peroxidation activity suggesting that eugenol might be able to donate hydrogen atoms in an aqueous environment more effectively than in lipid. On the contrary, β-caryophyllene reacts with peroxyl radicals better than donated hydrogen atoms. This might be due to its oil soluble property. Furthermore, the tyrosinase inhibition of clove oil and eugenol appeared to have the same extent whereas that of β-caryophyllene was approximately twice less than that of clove oil and eugenol. All tested oils were significantly less effective than kojic acid. The correlation among free radical scavenging activity, lipid peroxidation inhibition and tyrosinase inhibition of clove oil is illustrated in Figure 1 and the correlation coefficient between percent antioxidant index and percent inhibition of mushroom tyrosinase enzyme is shown in Table 3. At the same concentrations, inhibition of tyrosinase enzyme is more pronounced than antioxidant activity. When the correlations between percent tyrosinase inhibition and antioxidant index using DPPH test and TBARs of the main components were examined, it was found that tyrosinase inhibition of clove oil was less correlated with antioxidant indices. These findings are supported by the study of Arung et al. [21] that eugenol and clove buds oil can inhibit melanin formation in B16 melanoma cells. In addition, the results also suggest that antityrosinase of clove oil and its components may involve not only antioxidative action but also other mechanism of actions[22-23]. However, antioxidative activity of clove buds oil may be used in a skin-lightening agent based on the hypothesis that the skin pigmentation occurs by photo-oxidation of pre-existing melanin, the oxidative effect of reactive oxygen species in the skin which is induced by UV radiation and the oxidative polymerization of melanin intermediates by tyrosinase which is a copper-containing enzyme [3].

Table 3: Correlation between antioxidant activity and tyrosinase inhibition

Substance		DPPH	TBAR
Clove oil	DPPH	1	.804**
	TYROSINASE	.576*	.421*
Eugenol	DPPH	1	.639*
	TYROSINASE	.386*	.757**
Trans-caryophyllene	DPPH	1	.922**
	TYROSINASE	.923**	.960**

**Correlation is significant at the 0.01 level;

* Correlation is significant at the 0.05 level

Table 4: Cytotoxicity of clove oil and its major components against skin human fibroblasts

Substance	IC_{50} of SRB cytotoxicity test (µl/ml)		
Clove oil	0.162	±	0.005
Eugenol	0.167	±	0.003

β-caryophyllene	> 2.0

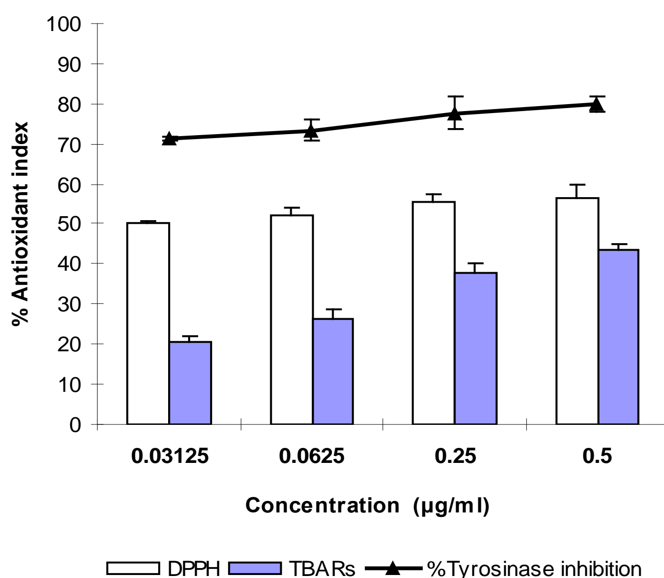

Fig. 1: Correlation between antioxidant index and tyrosinase inhibition of clove buds oil.

Table 4 demonstrates that β-caryophyllene was well tolerate at very high concentration without any toxic effect to dermal fibroblasts while clove oil and eugenol exhibited the cytotoxicity to fibroblasts at IC_{50} of 0.162 µl/ml and 0.167 µl/ml, respectively. However, The IC_{50} of clove oil and eugenol in this finding were less cytotoxicity than the study of Prashar et al.[24], whereas β-caryophyllene did not exhibit cytotoxic activity. This might be due to the method of testing as well as the different source of tested oils. In addition, according to the study of Arung et al.[21], clove oil and eugenol at a concentration of 100µg/ml exhibited melanin inhibition with less cytotoxicity towards B16 melanoma cells while β-caryophyllene did not inhibit melanogenesis and was toxic to the cells. As shown in Figure 2, at all tested concentrations, the oil treated cells significantly stimulated collagen synthesis greater than the untreated cells. Clove oil, eugenol and β-caryophyllene possessed a maximum collagen synthesis at the concentrations as low as 0.0156, 0.0625, and 0.0031µl/ml, respectively. As the oil concentrations increased greater than those, the amount of collagen gradually decreased. Although the fibroblasts were treated with the oils at the concentrations higher than their IC_{50} values of cytotoxicity test, the collagen synthesis in the treated groups were significantly greater than in control group. As reported by Khorshid et al.[25], the similar findings were also observed in essential oil of *Plectranths tenuiflorus* and thymol. In addition, the wound healing effect of these ethanolic solutions in rat wound model was also demonstrated.

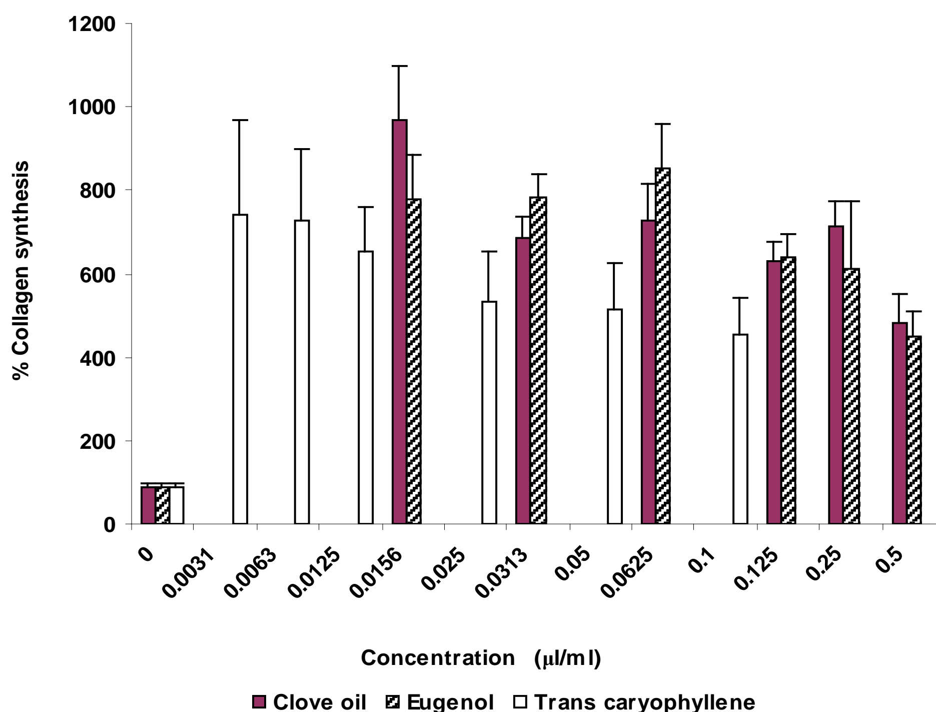

Fig. 2: Effect of clove oil and eugenol at various concentrations on collagen synthesis

CONCLUSION

Clove oil and eugenol might be a potential anti-aging substance by prevent aging of skin via oxidative processes and inducing collagen synthesis. Moreover, they also possess antityrosinase activity, suggesting a skin whitening effect. Besides other findings on their antimicrobial effects particularly antibacterial, antifungal and anti-acne, other activities on inhibition of 5-Lipoxygenase as well as formation of LTC4 in human PMNL suggesting anti-inflammatory action have also been reported[26-27]. Therefore, these properties of clove oil might have good implications for skin care applications. However, due to its cytotoxicity, clove buds oil should be used in very low concentrations with care and clinical trial should be further investigated.

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