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

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Received: 3 Feb 2012, Revised and Accepted: 16 March 2012

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 IC50 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. In addition, photaging 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 aging1-3. However, sunlight can induce skin tanning by ROS which may activate tyrosinase enzyme to catalyze the hydroxylation of monophenols to 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 pigments3-4. 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-mutagenic10, anti-inflammatory and antityrosinase 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-amino propane) dihydrochloride (ABAP) were obtained from Sigma Aldrich (Switzerland), calcium chloride (CaCl2), L-Tyrosine, Mushroom tyrosinase enzyme, 2, 2-diphenyl-1-picyrylhydrazyl (DPPH), tri-base buffer, sulforhodamine B (SRB) were purchased from Fluka, Switzerland. All culture media and culture supplements were purchase from Gibithai (Thailand).

Identification and quantification of clove oil components

GC/MS analysis of the essential oil sample (1% v/v in Dichloromethane; 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 1:10 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 m.s 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 concentrations of either reference compounds or clove oil and their peak area.

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

The free radical-scavenging activity of essential oil was measured according to the method modified from Lertasitthananon et al. 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 50 µl 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. a tocopherol acetate (vitamin E acetate) and butylated hydroxyl toluene (BHT) were used as the positive controls while ethanol alone served as a negative control.

Antioxidant Index (%) = \[1 - \left( \frac{A_{cont} - A_{blank}}{A_{cont}} \right) \times 100 \]
**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%) (Table 1). 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. 15.

**Table 1: The main components of clove oil**

<table>
<thead>
<tr>
<th>Chemical formula</th>
<th>Component</th>
<th>RI</th>
<th>Percent Area</th>
<th>Mode of Identification*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C10H13O2</td>
<td>eugenol</td>
<td>1350</td>
<td>99.16</td>
<td>a, b, c</td>
</tr>
<tr>
<td>C15H16</td>
<td>β-caryophyllene</td>
<td>1416</td>
<td>0.30</td>
<td>a, b, c</td>
</tr>
</tbody>
</table>

*a = mass spectra; b = RI; c = authentic compounds
Table 2: Bioactivities of clove oil and its major components compared with reference standards

<table>
<thead>
<tr>
<th>Substance</th>
<th>DPPH</th>
<th>TBARs</th>
<th>Tyrosinase Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clove oil</td>
<td>134.2±4.9</td>
<td>94.0±49.6</td>
<td>9.6±1.5</td>
</tr>
<tr>
<td>Eugenol</td>
<td>145.2±44.3</td>
<td>392.0±35.4</td>
<td>8.2±4.2</td>
</tr>
<tr>
<td>β-caryophyllene</td>
<td>1044.9±1067.3</td>
<td>4917.8±60.8</td>
<td>17.0±2.2</td>
</tr>
<tr>
<td>BHT</td>
<td>84.4±25.6</td>
<td>63.1±15.6</td>
<td>ND</td>
</tr>
<tr>
<td>Vitamin E acetate</td>
<td>4133.2±7984.7</td>
<td>3651.5±395.4</td>
<td>ND</td>
</tr>
<tr>
<td>Kojic acid</td>
<td>ND</td>
<td>ND</td>
<td>0.007±0.001</td>
</tr>
</tbody>
</table>

**Correlation is significant at the 0.01 level; 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. 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, antrical radical action of eugenol was less effective than that of BHT. The results can be explained as proposed by Bondet et al. 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. 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. However, 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.

Table 3: Correlation between antioxidant activity and tyrosinase inhibition

<table>
<thead>
<tr>
<th>Substance</th>
<th>DPPH</th>
<th>TBAR</th>
<th>Tyrosinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clove oil</td>
<td>1.0</td>
<td>1.0</td>
<td>0.804**</td>
</tr>
<tr>
<td>Eugenol</td>
<td>0.576*</td>
<td>0.421*</td>
<td></td>
</tr>
<tr>
<td>β-caryophyllene</td>
<td>0.386*</td>
<td>0.757**</td>
<td></td>
</tr>
<tr>
<td>Trans-caryophyllene</td>
<td>0.923**</td>
<td>0.960**</td>
<td></td>
</tr>
</tbody>
</table>

**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

<table>
<thead>
<tr>
<th>Substance</th>
<th>IC_{50} of SRB cytotoxicity test (µl/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clove oil</td>
<td>0.162 ± 0.003</td>
</tr>
<tr>
<td>Eugenol</td>
<td>0.167 ± 0.003</td>
</tr>
</tbody>
</table>

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Table 4 demonstrates that β-caryophyllene was well tolerated at very high concentration without any toxic effect to dermal fibroblasts while clove oil and eugenol exhibited the cytotoxicity to fibroblasts at IC₅₀ of 0.162 µl/ml and 0.167 µl/ml, respectively. However, the IC₅₀ 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 IC₅₀ 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 Plectranthus tenuiflorus and thymol. In addition, the wound healing effect of these ethanolic solutions in rat wound model was also demonstrated.
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 anti-tyrosinase 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 cells suggesting anti-inflammatory activity have also been reported\(^{14-17}\). 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.

ACKNOWLEDGEMENT

This study was financially supported by the Graduate School, Khon Kaen University, Thailand.

REFERENCES