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出版情報：Fitoterapia. 82 (2), pp.198-202, 2011-03. Elsevier
バージョン：

Inhibitory components from the methanol extract of the buds of clove (*Syzygium aromaticum*) on melanin formation in B16 melanoma cells

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Abstract

In the course to find a new whitening agent, we evaluated an inhibitory effects of methanol extract from bud of clove (*Syzygium aromaticum*) on melanin formation in B16 melanoma cells. The active compounds, eugenol and eugenol acetate showed melanin inhibition of 60% and 40% in B16 melanoma cell with less cytotoxicity at the concentration of 100 and 200 μg/mL, respectively.

Keywords : eugenol, eugenol acetate, *Syzygium aromaticum*, B16 melanoma cells, melanin inhibition

Introduction

Melanocytes are specialized cells in the skin that find their embryonic origin at the neural crest. During embryonic development, melanoblasts migrate to reach the basal layer of the epidermis where they differentiate to mature melanocytes possessing the complete machinery to ensure melanin synthesis and distribution within the skin. Melanin synthesis takes place within specialized intracellular organelles named melanosomes [1].

Melanin may be overproduced due to chronic sun exposure, melasma, or other hyperpigmentation diseases. Therefore, a number of depigmenting agents have been developed for cases of undesirable skin discoloration [2]. Up to now, most research on the regulation of melanogenesis has focused on the factors affecting tyrosinase, which catalyzes the rate-limiting step of the melanin biosynthesis pathway, specifically, the conversion of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and subsequently to DOPA quinone. Kojic acid [3] and arbutin [4] are known as tyrosinase inhibitors and are

used as skin-whitening cosmetics.

In Indonesia, where herbal medicine has been popular, more than 1300 species are known as medicinal plants, called Jamu [5]. The uses of Jamu fall into four categories of medicine: health care, beauty care (cosmetics), tonics, and bodily protection [6]. The use of traditional medicines has increased in recent years, and provides an interesting, largely unexplored source for the development of potential new drugs.

The clove tree (*Syzygium aromaticum*) named as “Cengkeh” in Indonesian was first cultivated on some islands of the Moluccas, Indonesia. In Southeast Asia, however, the clove is not much used for flavour food; medicinal use of both the clove (the flower bud) and the mother-of-clove (the fruit) has predominated. Cloves suppress toothache and halitosis; they are also a stimulant and carminative. Now, more than 90% of the cloves are used along with tobacco to produce 'kretek' cigarettes, which are smoked mainly in Indonesia [7]. The *S. aromaticum* is an evergreen tree and cloves, clove oil and oleoresin are commercial products. It is native to Molucca Island of Indonesia. The major clove-producing countries are Indonesia, Tanzania, Sri Lanka, Madagascar and, on a limited scale, India. In India it is grown in Kerala, Tamilnadu, Karnataka, Andaman and Nicobar Island over an area of 1735 hectares. The stem, unopened buds and leaves are normally used for extraction of essential oil. Owing to various kind of biological activities, clove oil finds extensive use in dental formulations, tooth paste, breath freshner, mouth washes, soaps, cosmetic items and insect repellent [8].

In present study, we evaluated the melanin biosynthesis inhibitory effect of the methanol extract from the buds of *S. aromaticum* on B16 melanoma cells in order to identify potential depigmenting agents such as skin-whitening cosmetics.

Experimental

Reagents

Eugenol, NaOH and DMSO were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Eugenol and eugenol acetate were from TCI (Tokyo Chemical Industry, Tokyo, Japan). The 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) from Sigma (St. Louis, MO), EMEM from Nissui Chemical Co (Osaka, Japan). Essential oil of clove was purchased from GAIA Essential Oil. The Ethylenediaminetetraacetic acid (EDTA) from Dojindo Co, (Kumamoto, Japan). Other chemicals are of the highest grade commercially available.

Plant material

The bud of clove (*S. aromaticum*) was collected from traditional market in Samarinda, East Kalimantan, Indonesia on July, 2009. The voucher specimen was deposited in Wood Chemistry Laboratory, Department of Forest Product Technology, Faculty of Forestry, Mulawarman University.

Preparation of plant extract

The dried bud of clove (14.9 g) was extracted with methanol at room temperature for 24 h. The extract solution was filtered and concentrated *in vacuo*, to obtain the crude methanol extract (5.9 g).

Isolation of eugenol and eugenol acetate

Methanol extract of *S. aromaticum* (1.02 g) that showed potent inhibitory effect of melanin production in B16 melanoma cells, was separated by silica gel column (800 g

of Wakogel C-200, 3.5 x 60 cm) and eluted with *n*-hexane/EtOAc [10:0 (150 mL), 9:1 (100 mL), 8:2 (100 mL), 7:3 (200 mL), 6:4 (200 mL), 5:5 (100 mL), 4:6 (400 mL), 3:7 (200 mL), 2:8 (100 mL), 1:9 (100mL)] and EtOAc/MeOH [9:1 (100 mL), 7:3 (100 mL), 5:5 (200 mL), 3:7 (200 mL), 1:9 (100 mL), 0:10 (850 mL)] to give fifty six fractions (Fr 1 to Fr 56). Fraction 4 (184.3 mg) was oily, the highest content and gave pleasant aroma. By using GC-MS, this fraction was analyzed and compared with the standard compounds such as eugenol and eugenol acetate.

GC-MS analysis

Fraction 4 and Essential oil of clove was dissolved in acetone and subjected to qualitative analysis by using GC-MS instrument (GC-17A, QP-5050). The instrument equipped with a column : DB-5 (30 m × 0.25 mm i.d., 0.25 μm film thickness, J & W Scientific Inc.), split ratio : 1:50, and running with temperature program : INJ 250°C DET 250 ^{o}C, 50 ^{o}C at 3 ^{o}C /min. hold to 250 ^{o}C at 7 ^{o}C /min and 250 ^{o}C at 10 ^{o}C /min. hold.

Cell culture

A mouse melanoma cell line, B16, was obtained from RIKEN Cell Bank. The cells were maintained in EMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.09 mg/mL theophylline. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Inhibitory effect of melanin biosynthesis and cell viability using cultured B16 melanoma cells

This assay was determined as described by Arung et al [9]. Briefly, confluent cultures of B16 melanoma cells were rinsed in phosphate-buffered saline (PBS) and removed from the plastic using 0.25 % trypsin/EDTA. The cells were placed in two plates of 24-well plastic culture plates (1 plate is for determining of melanin and other is for cell viability) at a density of 1×10^5 cells/well and incubated for 24 h in media prior to being treated with the samples. After 24 h, the media were replaced with 998 μL of fresh media and 2 μL of DMSO was added with or without (control) the test sample at various concentrations (n=3) and arbutin was used as a positive control. The cells were incubated for an additional 48 h, and then the medium was replaced with fresh medium containing each sample. After 24 h, the remaining adherent cells were assayed (see below).

Determination of melanin content in B16 melanoma cells

The melanin content of the cells after treatment was determined as follows. After removing the medium and washing the cells with PBS, the cell pellet was dissolved in 1.0 mL of 1*N* NaOH. The crude cell extracts were assayed using a micro plate reader (Bio-Tek, USA) at 405 nm to determine the melanin content. The results from the cells treated with the test samples were analyzed as a percentage of the results from the control culture.

Cell viability

Cell viability was determined by use of the micro culture tetrazolium technique

(MTT). The MTT assay provides a quantitative measure of the number of viable cells by determining the amount of formazan crystals produced by metabolic activity in treated versus control cells. Culture was initiated in 24-well plates at 1×10^5 cells per well. After incubation, 50 μL of MTT reagent [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2*H*-tetrazolium bromide in PBS (5 mg/mL)] was added to each well. The plates were incubated in a humidified atmosphere of 5% of CO_2 at 37°C for 4 h. After the medium was removed, 1.0 mL isopropyl alcohol (containing 0.04 N HCl) was added into the plate, and the absorbance was measured at 570 nm relative to 630 nm.

Results and discussion

Clove oil (*S. aromaticum*) is widely used as a perfume and food flavoring, as a medicine for the treatment of asthma and various allergic disorders in Korea and as a general antiseptic in medical dental practices. The clove oil might also be used as an chemopreventative agent [10]. Srivastava, et al. [8] reported that clove oil has some properties such as anthelmintic, analgesic, antibacterial, antifungal and anticarcinogenic.

In present study, we evaluate anti melanogenesis property of the methanol extracts of the buds of clove. The methanol extracts were assayed by using B16 melanoma cells in order to evaluate the inhibition of melanin formation and cell viability. In Figure 1, the inhibition of methanol extracts of clove on melanin formation in B16 melanoma cells was shown at various concentrations. At the concentration of 50 μg/mL, the methanol extract of clove showed potent melanin formation inhibitory activity more than 40% with less cytotoxicity. The similar result was depicted by arbutin, as positive control.

Based on this result, we separated the methanol extract by using silica gel column

fractionation in order to find the active compounds. This separation, gave 56 fractions and fraction 4 (C-4) was oily, high content and pleasant smell. Therefore, we focused on fraction C-4 to evaluate its anti melanogenesis effect. In Figure 2, C-4 showed melanin inhibition on B16 melanoma cells about 25% with less cytotoxicity at 100 μg/mL of concentration. By GC-MS analysis (Figure 3), we have compared the standard compound, such as eugenol and eugenol acetate with fraction C-4. The GC-MS data of standards obviously indicated that fraction C-4 contained eugenol and eugenol acetate (Figure 4). Eugenol was the main compound in this fraction (Figure 3). It had been reported that the essential oil obtained by hydro distillation of buds of clove, contained eugenol and β-caryophyllene as dominant compounds [8, 11, 12]; eugenol and eugenol acetate as abundant compounds [13]. In methanol extract of bud of clove, Son et al, [14] reported that eugenol was isolated with some phenolic compounds.

Next, we evaluated the effect of eugenol and eugenol acetate which were dominantly contained in oily fractions on melanin formation in B16 melanoma cells as shown in Figure 5 and 6. Both eugenol and eugenol acetate showed the inhibitory activity of melanin formation dose dependently. Eugenol inhibited melanin formation more than 42% with less cytotoxicity (5%) at 100 μg/mL but high concentration, at 200 μg/mL, it showed cytotoxicity of 23%. Eugenol acetate attenuated melanin formation about 40% with less cytotoxicity (14%) at 200 μg/mL and depicted cytotoxicity effect of 71% at 250 μg/mL. In addition, we tested the melanin formation of essential oil of clove in order to compare the effect on it. In Figure 7, it depicted the effect of essential oil of clove which showed cytotoxicty on B16 melanoma cell rather melanin formation inhibition. The presence of β-caryophyllene and isoeugenol in essential oil of clove may cause the cytotoxicity effect as shown in Figure 8. In our knowledge, this is the first

report that methanol extract from bud of clove, eugenol and eugenol acetate exihibited melanin inhibition in B16 melanoma cells.

In conclusion, eugenol and eugenol acetate are promising compounds that could be useful for treating hyperpigmentation, as a skin-whitening agent with pleasant smell. However, it should be noted that safety is a primary consideration for its practical use in humans.

Acknowledgement

This research supported by Japan Society for the Promotion of Science (JSPS) for Postdoctoral Fellow of Foreign Researchers.

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Figures Legend

Figure 1. Effect of methanol extracts of the buds of clove (*S. aromaticum*) on melanin formation in B16 melanoma cells. Each column represents the mean ± SD, with n = 3 (Student's *t*-test). Significant different from the control value : P<0.05 (*), P<0.01 (**).

Figure 2. Effect of Fraction C-4 on melanin formation in B16 melanoma cells [Arbutin 100 = 100 μg/mL]. Each column represents the mean ± SD, with n = 3 (Student's *t*-test). Significant different from the control value : P<0.05 (*), P<0.01 (**).

Figure 3. GC-MC analysis of Fraction C-4 of methanol extracts of the buds of clove (*S. aromaticum*).

Figure 4. Structure of eugenol and eugenol acetate

Figure 5. Effect of eugenol on melanin formation in B16 melanoma cells [Arbutin 100 = 100 μg/mL]. Each column represents the mean ± SD, with n = 3 (Student's *t*-test). Significant different from the control value : P<0.05 (*), P<0.01 (**).

Figure 6. Effect of eugenol acetate on melanin formation in B16 melanoma cells [Arbutin 100 = 100 μg/mL]. Each column represents the mean ± SD, with n = 3 (Student's *t*-test). Significant different from the control value : P<0.05 (*), P<0.01 (**).

Figure 7. Effect of essential oil of clove on melanin formation in B16 melanoma cells [Arbutin 100 = 100 μg/mL]. Each column represents the mean ± SD, with n = 3 (Student's *t*-test). Significant different from the control value : P<0.01 (**).

Figure 8. GC-MC analysis of essential oil of clove (*S. aromaticum*).

Figure 1.

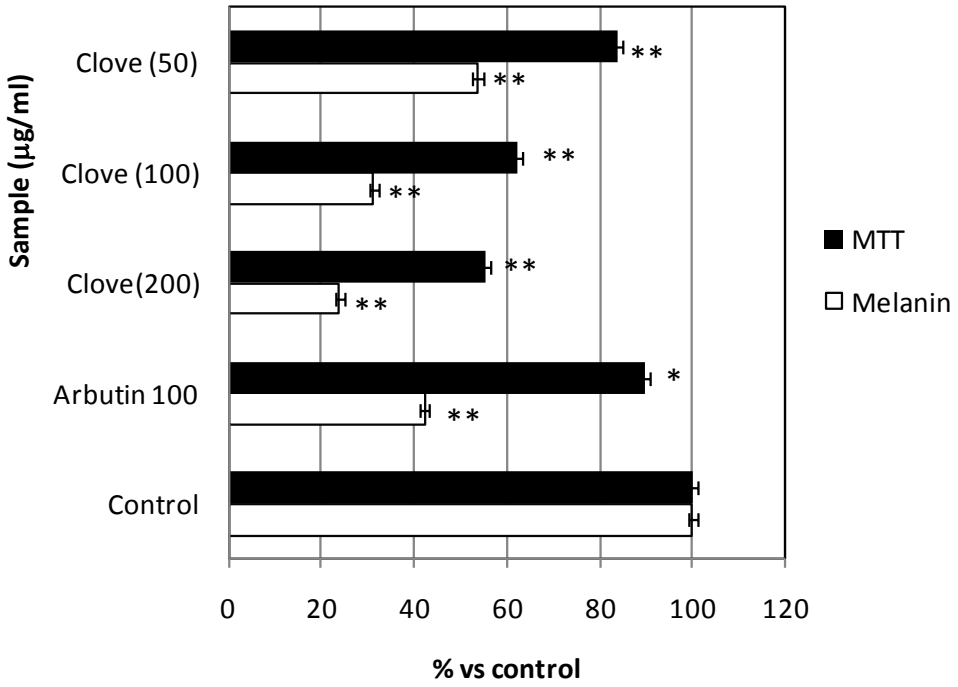

Sample (µg/ml)
Clove (50)
Clove (100)
Clove(200)
Arbutin 100
Control
**
**
**
**
**
**
*
**
MTT
Melanin
0
20
40
60
80
100
120
% vs control

Figure 2.

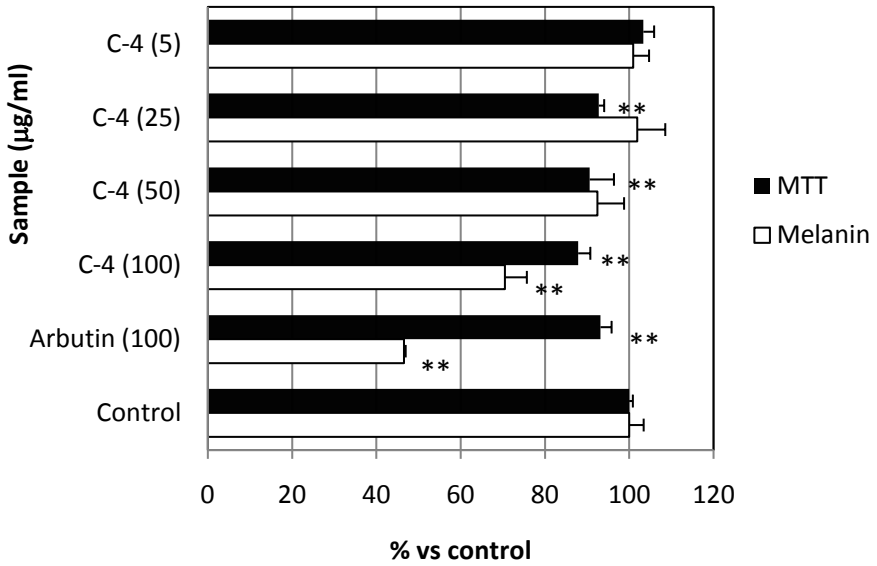

Sample (μg/ml)
C-4 (5)
C-4 (25)
C-4 (50)
C-4 (100)
Arbutin (100)
Control
**
**
**
**
**
MTT
Melanin
0
20
40
60
80
100
120
% vs control

Figure 3.

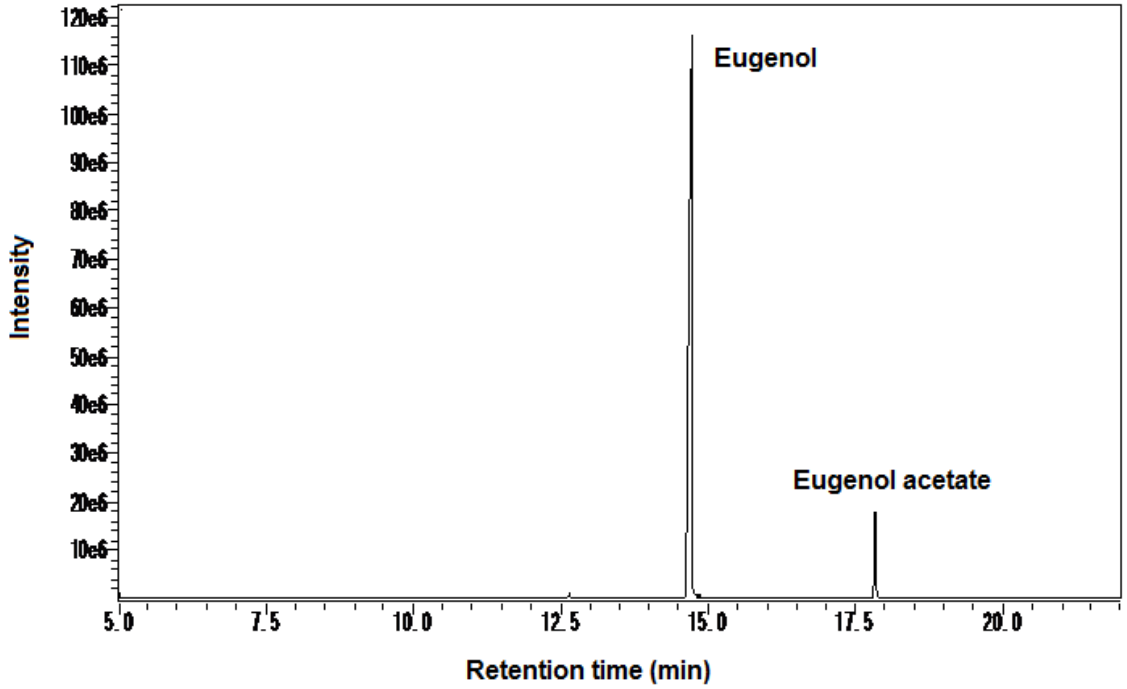
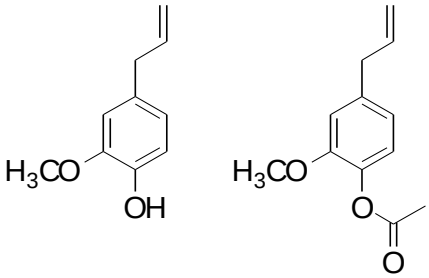

Eugenol
Eugenol acetate
Intensity
120e6
110e6
100e6
90e6
80e6
70e6
60e6
50e6
40e6
30e6
20e6
10e6
5.0
7.5
10.0
12.5
15.0
17.5
20.0
Retention time (min)

Figure 4.



Eugenol Eugenol acetate

Figure 5.

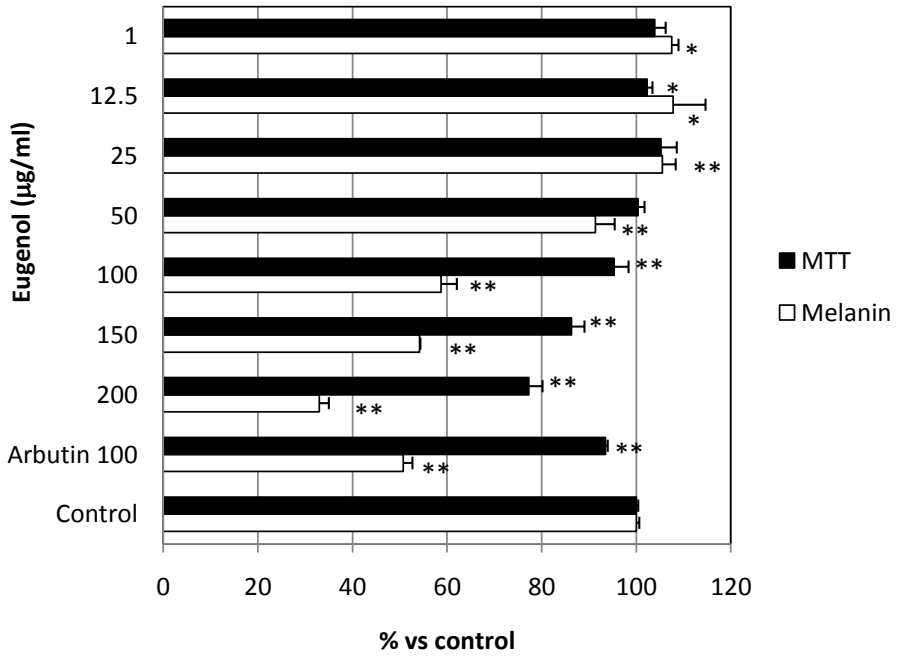

Eugenol (µg/ml)
1
12.5
25
50
100
150
200
Arbutin 100
Control
0
20
40
60
80
100
120
% vs control
MTT
Melanin

Figure 6.

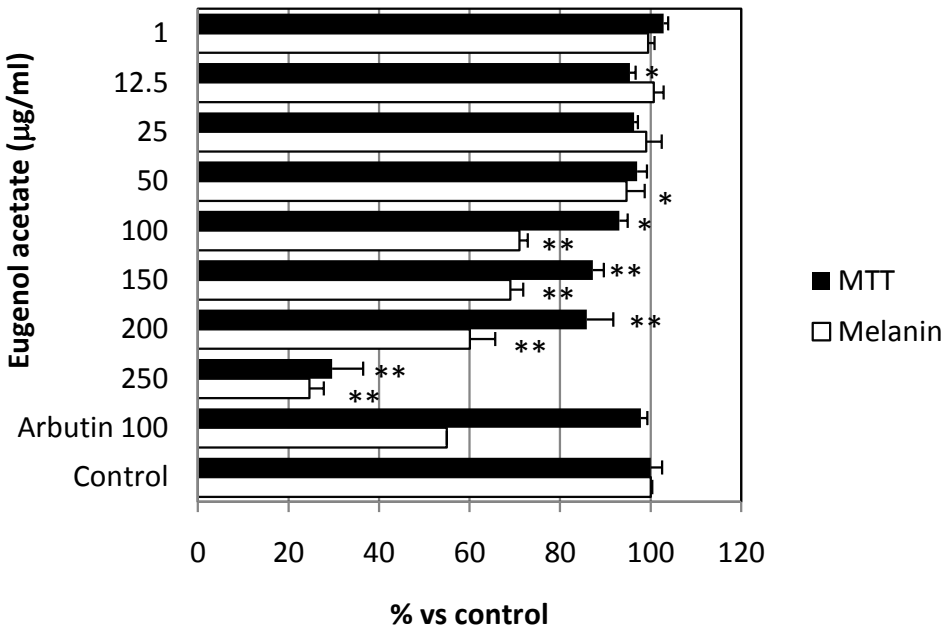

Eugenol acetate (µg/ml)
1
12.5
25
50
100
150
200
250
Arbutin 100
Control
0
20
40
60
80
100
120
% vs control
MTT
Melanin

Figure 7.

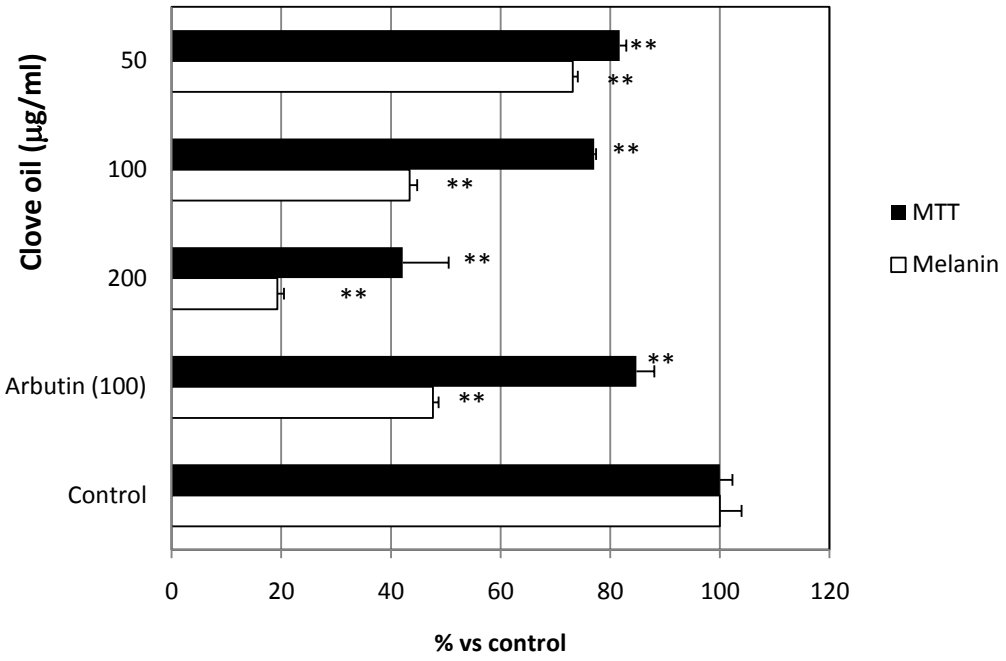
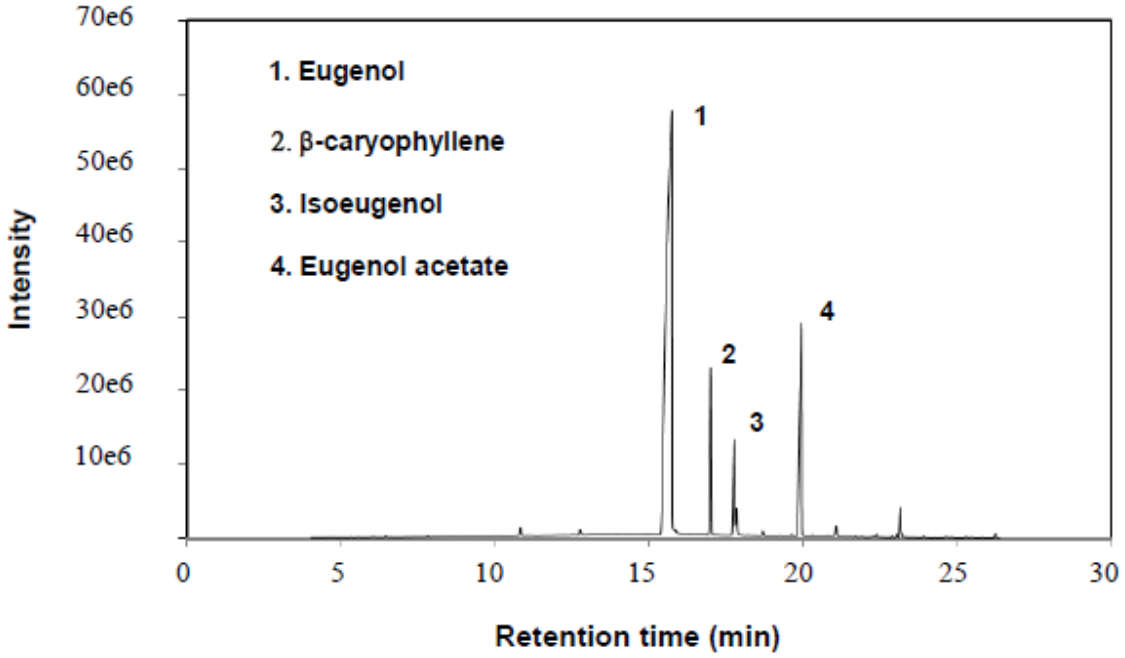

Clove oil (µg/ml)
50
100
200
Arbutin (100)
Control
**
0
20
40
60
80
100
120
% vs control
MTT
Melanin

Figure 8.


70e6
60e6
50e6
40e6
30e6
20e6
10e6
Intensity
1. Eugenol
2. β-caryophyllene
3. Isoeugenol
4. Eugenol acetate
1
2
3
4
0
5
10
15
20
25
30
Retention time (min)