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Original Article

Comparative anti-inflammatory activity of eugenol and eugenyl acetate on the murine immune response *in vitro*

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Abstract

The utility of eugenol, a naturally occurring phenolic compound, for various applications is limited due to its unfavorable properties. Thus, eugenyl acetate is under consideration as a substitute. The immunomodulatory activity of eugenyl acetate compared to eugenol was investigated. Eugenyl acetate stimulated oxidative burst release from macrophages much more than eugenol. Eugenol and eugenyl acetate similarly suppressed B-cell proliferation through the T-cell independent pathway, the same as lipopolysaccharide, as well as the T-cell dependent pathway, the same as pokeweed mitogen. Eugenol effectively stimulated T-cell proliferation more than eugenyl acetate through the same pathway as concanavalin A. Moreover, both eugenol and eugenyl acetate showed similar maximal activity in inhibiting interferon γ (IFN-γ) and interleukin-2 (IL-2) production and stimulating IL-10 secretion. Our findings suggested that eugenyl acetate might be used as a substitute for or accompany eugenol as a potential therapeutic agent in treating inflammatory diseases and thereby decrease the potential high dose toxicity of eugenol.

Keywords: eugenol, eugenyl acetate, macrophage phagocytosis, splenocyte proliferation, cytokine

1. Introduction

Nowadays, the use of medicinal plants as alternative therapy for treatment of diseases is increasing. Various investigations use natural sources such as plant extracts, essential oils, chemical compounds contained in plants, natural molecular fingerprints or their modified structures to study their biological activities. Eugenol (2-methoxy-4-(2-propenyl) phenol) is a naturally occurring phenolic compound in basil, cinnamon, and nutmeg (Raghavenra *et al*., 2006), and it is the major component (80–95%) of clove oil (Szabadics & Erdelyi, 2000). It is widely used as a flavoring agent in food products, fragrance in cosmetics (Chang *et al*., 2002; Tai *et al*., 2002), and in pharmaceutical applications (Pramod *et al*., 2010). Several pharmacological activities of eugenol have been reported that include anticonvulsant (Dallmeier & Carlini, 1981), anti-stress (Sen *et al*., 1992), anti-leishmania (Ueda-Nakamura *et al*., 2006), antinociceptive (Dal Bó *et al*., 2013), anti-inflammatory, anti-microbial, and anti-tumor, and it is also used as a local anesthetic (Pramod *et al*., 2010).

Unfortunately, applications of eugenol are limited due to its poor water solubility, chemical instability when exposed to light or high temperature, and the requirement for a high dose to achieve a therapeutic effect (Mastelić *et al*., 2008; Shimoda *et al*., 2006), which may cause high dose toxicity (Markowitz *et al*., 1992). At concentrations higher than 3 mM, eugenol was cytotoxic to oral mucosal fibroblasts and decreased cellular adenosine triphosphate. The acute oral LD_{50} of eugenol was 2.13 g/kg in guinea pigs, 2.68 g/kg in rats, and 3.0 g/kg in mice (Tisserand & Young, 2014). The chemical properties of eugenol and eugenyl acetate were studied using density functional theory and Hartree-Fock methods. It had been found that eugenyl acetate was more stable than eugenol

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which is unstable. However, eugenol may reduce the growth of cancer cells by reacting with the free radicals associated with cancer (Gökalp, 2016). Moreover, both clove oil and its esterified product, eugenyl acetate, presented antimicrobial activities against gram-negative bacteria. They both had high antioxidant potential, particularly for eugenyl acetate (Vanin *et al*., 2014). Therefore, eugenol derivatives such as eugenyl acetate were considered in this study. Similar to eugenol, eugenyl acetate can be extracted from many spicy herbs (Govinden-Soulange *et al*., 2004; Jirovetz *et al*., 2006) and has also been successfully synthesized by chemical process (Santos *et al*., 2009).

Inflammation is a complex biological response of tissues to pathogens, damaged cells and irritants (Ferrero-Miliani *et al*., 2007) that is modulated by cytokines. Macrophages respond to a variety of membrane stimulants by producing and then releasing extracellularly a number of reactive oxygen and nitrogen species to kill invading microorganisms by oxidative burst (Davicino *et al*., 2008; Symons & King, 2003). The anti-inflammatory activity or other immune-related activities of plant extracts and their naturally occurring compounds such as eugenol-rich plant extract or essential oil and eugenol have been investigated. An aqueous extract of clove inhibited macrophage production of IL1-γ and IL-6 in mice and essential oil of clove inhibited these cytokines *in vitro* (Rodrigues *et al*., 2009). A hexane fraction of *Cinnamomum tamala* Linn. in which eugenol was abundant showed anti-inflammatory properties in carrageenan-induced paw edema and lipopolysaccharide (LPS)-induced nitric oxide (NO) production in rat peritoneal macrophage cultures that paralleled its concentrations of eugenol and total phenolic content (Chaurasia & Tripathi, 2011). Moreover, eugenol inhibited the plaque-forming cell response of mouse splenocytes (Vishteh *et al*., 1986) and induced apoptosis in HL-60 human promyelocytic leukemia cells via reactive oxygen species (ROS) generation (Yoo *et al*., 2005). There was also a previous report on eugenol and eugenyl acetate inhibiting platelet aggregation (Srivastava *et al*., 1991) and acting as antioxidants (Ito *et al*., 2005; Lee & Shibamoto, 2001). Nevertheless, studies of the immunomodulatory activity of eugenyl acetate have been limited. Thus, we undertook a study of the immunological effects of eugenyl acetate compared to eugenol and the possibility of using eugenyl acetate as a substitute.

2. Materials and Methods

2.1 Materials

Eugenol, eugenyl acetate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), nitroblue tetrazolium (NBT) dye, *p*-nitrophenyl phosphate (*p*-NPP), phytohemagglutinin (PHA), concanavalin A (Con A), lipopolysaccharide (LPS), pokeweed mitogen (PWM), dimethyl sulfoxide (DMSO), phosphate buffer saline (PBS), phorbol-12-myristate-13-acetate (PMA), zymosan A and antibiotic-antimycotic solution (100 U penicillin, 100 µg streptomycin and 0.25 µg amphotericin B per mL) were purchased from Sigma-Aldrich (Deisenhofen, Germany). β–mercaptoethanol and Triton X-100 were purchased from Fisher Scientific (Loughborough, UK) and fetal bovine serum (FBS) and RPMI-1640 medium were purchased from GIBCO/BRL Invitrogen (Paisley, Scotland). Interferon-γ (IFN-γ), interleukin-2 (IL-2), and IL-10 ELISA kits were purchased from eBioscience Inc. (San Diego, USA).

2.2 Animals

Female ICR mice (5-6 weeks old) were obtained from the National Laboratory Animal Centre, Mahidol University, Bangkok, Thailand. The animals were housed under standard conditions at 25±2 °C and fed with standard pellets and tap water. The experiments were conducted under the surveillance of the Ethics Committee of Naresuan University, Phitsanulok, Thailand (Ethics approval number: 048010019).

2.3 Preparation of peritoneal mouse macrophages

Peritoneal macrophages were isolated following intraperitoneal injection of 1 mL FBS as a stimulant (Manosroi *et al*., 2003). Three days later, the peritoneal exudate was collected by peritoneal lavage with complete RPMI medium (RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 50 mM 2-mercaptoethanol and 1 mL of an antibiotic-antimycotic solution). The exudates from 3 mice were pooled and centrifuged at 300×*g* for 20 min at 25 °C and the cells were washed twice and re-suspended in RPMI medium. The purification of macrophages was performed using their ability to adhere to the tissue culture plastic surface by culturing for 2 h at 37 °C. After that, non-adherent cells were removed by gentle washing 3 times with RPMI. The adherent cells were rinsed vigorously with cRPMI and approximately 90-95% of the macrophages determined by morphologic criteria under microscope were obtained. The number of cells was adjusted to 1×10^6 cell/mL as determined by counting in a hemocytometer. Cell viability was tested by the trypan-blue dye exclusion technique both before and after treatment with tested concentrations of eugenol or eugenyl acetate.

2.4 Preparation of mouse splenocytes

Mice were euthanized by cervical dislocation and the spleens were removed aseptically. Cell suspensions were prepared by mincing and tapping the spleen fragments on a stainless steel 200-mesh screen in RPMI medium (Manosroi *et al*., 2003). The splenocytes from 2-3 mice were pooled. After centrifugation at 300×*g*, for 10 min at 37 °C, the cells were washed twice and re-suspended in complete RPMI medium. The number of cells was adjusted to 5×10^7 cell/mL by counting in a hemocytometer. Cell viability was tested by the trypan-blue dye exclusion technique both before and after treatment with tested concentrations of eugenol or eugenyl acetate.

2.5 Macrophage function assay

The NBT dye reduction assay was carried out as previously described (Rainard, 1986). Macrophages (1×10^5 cell/well) were treated with various concentrations of eugenol or eugenyl acetate for 24 h at 37 °C in a 5% CO_2 humidified incubator. Macrophages were incubated with zymosan A (5×10^6 particles/well) and 1.5 mg/mL of NBT dye. After incubation for 60 min, the adherent macrophages were rinsed vigorously with RPMI medium and washed four times with methanol. After air-drying, 2 M KOH and DMSO were added

and the absorbance was measured at 570 nm using a microplate reader (Bio-Tek Instrument Inc., Winooski, VT, USA).

The cellular lysosomal enzyme activity was used to quantify acid phosphatase in macrophages as previously described (Suzuki *et al*., 1988). Macrophage suspensions (1×10^5 cells/well) were treated with eugenol or eugenyl acetate for 24 h at 37 °C in a 5% CO_2 humidified atmosphere. The medium was removed by aspiration and 0.1% Triton X-100, 10 mM *p*-NPP solution and 0.1 M citrate buffer (pH 5.0) were added to each well. The cells were further incubated for 30 min after which 0.2 M borate buffer (pH 9.8) was added. The absorbance was measured at 405 nm using a microplate reader.

The stimulation index (SI) was calculated as the ratio of the optical density (OD) values of the sample and control.

2.6 Mitogen-induced splenocyte proliferation assay

The mitogen-induced splenocyte proliferation assay was carried out according to a previous report (Mosmann, 1983). Four mitogens (LPS, PWM, PHA and Con A) were used at optimum doses. Briefly, splenocyte suspensions were treated with eugenol or eugenyl acetate with simultaneous mitogen induction (5 μg/mL) for 48 h at 37 °C in a humidified 5% CO_2 atmosphere. After incubation, 5 mg/mL of MTT dye were added and the cells were incubated for a further 4 h. The culture medium was removed by aspiration, 0.04 M HCl in isopropyl alcohol and distilled water were added, and the absorbance at 570 nm was measured by a microplate reader. The SI was calculated as the ratio of the OD values of the sample and mitogen.

2.7 LPS-induced cytokine production assay

The productions of mouse IFN-γ, IL-2, and IL-10 were measured by ELISA according to the instructions of the manufacturer. Splenocytes were treated with extract plus LPS (5 μg/mL) for 48 h at 37 °C in a humidified 5% CO_2 incubator and the culture supernatants were analyzed. Briefly, a 96-well microtiter plate was pre-coated overnight with capture antibody. After blocking and several washings, working standards and samples were then added for incubation for 2 h. After washings, the working detector solution containing biotinylated anti-mouse cytokine monoclonal antibody and avidin-horseradish peroxidase conjugate was added to each well and incubated for 1 h. Substrate solution was then added, followed by the addition of stop solution, and the absorbance was read within 30 min using a microplate reader at 450 nm.

2.8 Statistical analysis

All experiments were performed in triplicate and the results are expressed as mean±S.E. Statistical differences between groups were assessed using a one-way analysis of variance followed by multiple comparisons using Tukey's method. The level of significance was set at $P<0.05$.

3. Results

The effects of eugenol and eugenyl acetate on the phagocytic activity of mouse macrophages were investigated using NBT dye reduction and lysosomal enzyme activity assays. Eugenol and eugenyl acetate were not toxic to the macrophages at the tested concentrations because cell viability was greater than 90%. The results indicated that eugenyl acetate at 1-1000 μg/mL stimulated NBT dye reduction in a non-dose dependent manner, reaching a maximum stimulation of about 124% (SI 2.2) at 10 μg/mL, while eugenol did not induce any change. Both eugenol (100 and 1000 μg/mL) and eugenyl acetate (1-1000 μg/mL) similarly diminished lysosomal enzyme activity approximately 30% (SI 0.7). The results are presented in Figure 1.

The impact of eugenol and eugenyl acetate on mitogen-induced splenocyte proliferation activity was evaluated using the MTT technique. Eugenol and eugenyl acetate were not toxic to splenocytes at the tested concentrations because cell viability was greater than 90%. In the presence of PHA, only eugenol at 0.1 μg/mL increased splenocyte proliferation, by about 34% (SI 1.3). All concentrations of eugenol with Con A similarly increased the stimulation of splenocyte proliferation by about 70% (SI 1.7), which was 40–50% higher than eugenyl acetate at 0.1–10 μg/mL (SI 1.3). In contrast, eugenol and eugenyl acetate suppressed the stimulation of splenocyte proliferation by about 40% (SI 0.6) when induced

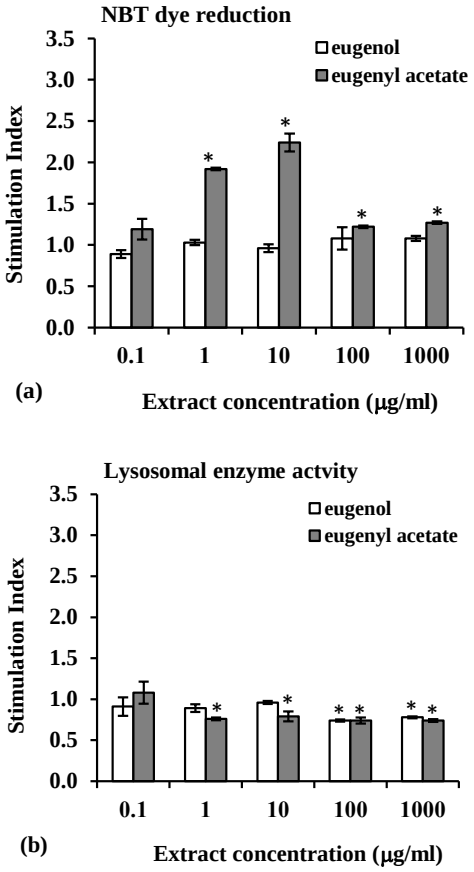

Figure 1. Effect of eugenol and eugenyl acetate on mouse macrophage phagocytosis using (a) NBT (nitroblue tetrazolium) dye reduction and (b) lysosomal enzyme activity assays. Each value represents the mean±S.E. of triplicate testing compared to the control, *; $P<0.05$.

by LPS and by 20–30% (SI 0.7-0.8) when induced with PW-M, respectively. The results are shown in Figure 2.

The production of IFN-γ, IL-2, and IL-10 from LPS induced splenocytes treated with eugenol and eugenyl acetate was studied using ELISA. Eugenol at 0.01–1000 μg/mL suppressed IFN-γ secretion considerably, reaching a maximum suppression of about 95% at 1000 μg/mL. This was also observed in the culture with eugenyl acetate at 1000 μg/mL. Likewise, cultures with eugenol at 1–1000 μg/mL or eugenyl acetate at 1000 μg/mL showed similar levels of inhibition of IL-2 production at about 40%. Additionally, eugenol at 100 or 1000 μg/mL and eugenyl acetate at 1–1000 μg/mL produced a tremendous increment in IL-10 production, with a maximal value of about 242% (1000 μg/mL) for eugenol and 263% (100 μg/mL) for eugenyl acetate. The results are presented in Figure 3.

4. Discussion

Eugenol is widely used as a flavoring agent in food products and as a fragrance in cosmetics (Chang *et al*., 2002; Tai *et al*., 2002). Various pharmacological activities of eugenol have been investigated (Park *et al*., 2000; Sen *et al*., 1992; Ueda-Nakamura *et al*., 2006). Due to an instability issue with eugenol, eugenol derivatives such as eugenyl acetate have been considered as a substitute. Eugenyl acetate is usually extracted from the same sources as eugenol but to a lesser extent (1–4%) (Govinden-Soulange *et al*., 2004; Jirovetz *et al*., 20 06). It can also be obtained by direct chemical modification of eugenol by esterification (Santos *et al*., 2009).

It is well known that macrophages play an important role in the defense mechanisms against host infection and the killing of tumor cells. Macrophages respond to a variety of membrane stimulants with production and extracellular release of a number of reactive oxygen and nitrogen species designed to kill invading microorganisms by oxidative burst (Davicino *et al*., 2008; Symons & King, 2003). In the present study, we evaluated the activity of eugenyl acetate compared to eugenol on macrophage phagocytic and oxidative burst activity. A greater reduction of NBT dye represented a higher activity of oxidase enzyme which reflected the stimulation of phagocytosis in proportion to intracellular killing (Rainard, 19 86). Our investigations showed that the level of NBT dye reduction generated by eugenol was no different from that in the control, while it increased markedly in the presence of eugenyl acetate. The non-dose response of eugenyl acetate was

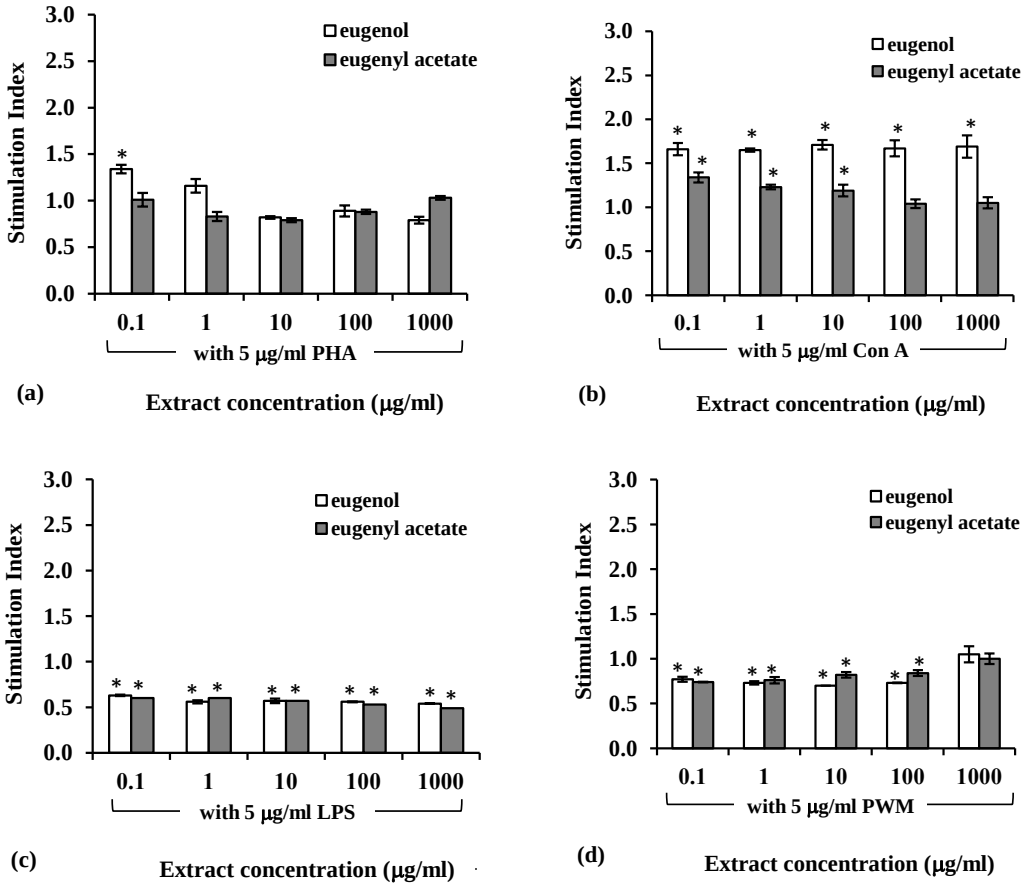

Figure 2. Effect of eugenol and eugenyl acetate on splenocyte proliferation in the presence of 5 μg/mL mitogen; (a) PHA (b) Con A (c) LPS, and (d) PWM. Each value represents the mean±S.E. of triplicate testing compared to each mitogen alone, *; P<0.05.

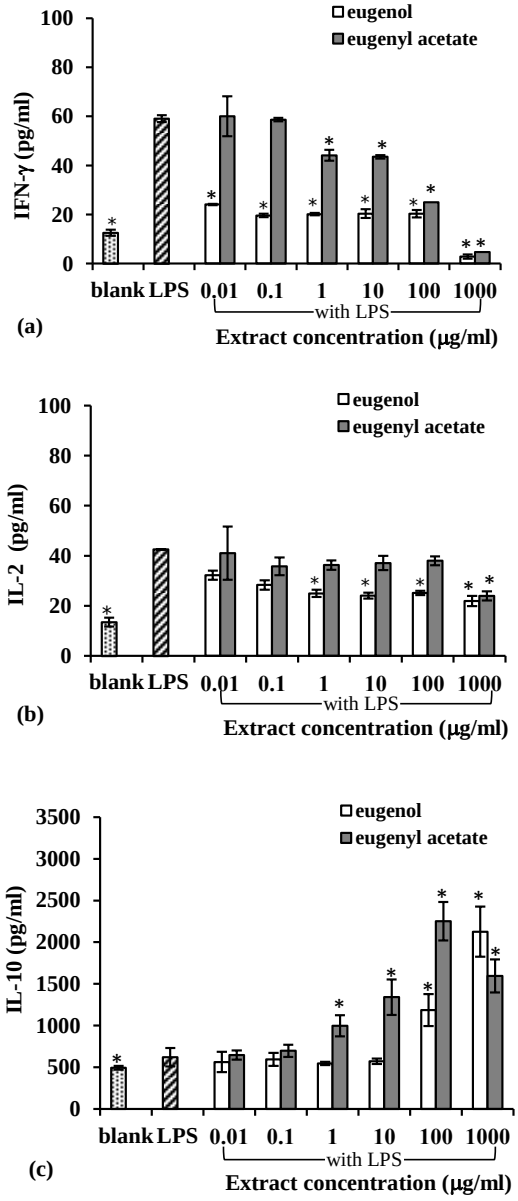

Figure 3. Effect of eugenol and eugenyl acetate on 5 µg/mL lipopolysaccharide (LPS)-induced cytokine production: (a) IFN-γ, (b) IL-2, (c) IL-10. Each value represents the mean±S.E. of triplicate testing compared to LPS, *; $P<0.05$.

possibly due to the high concentration which possibly disturbed macrophage function on oxidase enzyme production through various mechanisms (Lim *et al.*, 2007; Maraldi, 2013). Therefore, a decrease of NBT dye reduction due to a lowering of oxygen radical production was observed. However, eugenol and eugenyl acetate were not toxic to the macrophages at the tested concentrations because cell viability was more than 90%. These results indicated the effectiveness of eugenyl acetate in stimulating macrophage phagocytosis by oxidative burst reduction. In this investigation, we proposed to study the effects of eugenol and eugenyl acetate at the concentration range for food. The recommended limits vary depending on the type of food. The highest concentrations indicated 25 ppm for meat products and the lowest was 2.83 ppm for non-alcoholic beverages. However, the lower concentration range of eugenyl acetate might be further studied to clarify the dose response relationship of eugenyl acetate on oxidative burst reduction activity.

With regard to lysosomal enzyme activity, transformation of *p*-NPP to a colored compound by the acid phosphatase of stimulated macrophages is correlated to degranulation in phagocytosis (Suzuki *et al.*, 1990). Both eugenol and eugenyl acetate decreased lysosomal enzyme activity slightly which suggested weak inhibition of acid phosphatase production. Previous investigations demonstrated that eugenol induced ROS, leading to apoptosis in human promyelocytic (HL-60) leukemia cells (Yoo *et al.*, 2005), but reduced nicotine-induced ROS in murine macrophages (Mahapatra *et al.*, 2011). Different concentrations, cell sources, and stimulants could be responsible for the apparent discrepancies. Since eugenyl acetate was able to improve macrophage phagocytesis beyond that induced by eugenol in the present study, it can be assumed that the acetate group in eugenyl acetate confers some advantage for pro-phagocytic activity.

The colorimetric MTT method, which has several desirable advantages for assaying cell survival and proliferation, was used to assay splenocyte proliferation in this study. MTT is cleaved by all living, metabolically active cells and the amount of MTT formazan generated is directly proportional to the cell number (Mosmann, 1983). The presence of mitogens in the system can illuminate the possible activation pathway of extracts. LPS and PWM were used to stimulate B-cell proliferation through T-cell independent and T-cell dependent pathways while PHA and Con A were used to activate T-cell proliferation in different subtypes (Bekeredjian-Ding *et al.*, 2012; Nakamura *et al.*, 1986).

Our results showed that both eugenol and eugenyl acetate decreased splenocyte proliferation to a similar degree by about 40–50% in the presence of LPS and 30% in the presence of PWM. This suggested that both eugenol and eugenyl acetate moderately suppressed B cell proliferation through the T-cell independent pathway, the same as LPS, and slightly inhibited B cell proliferation through the T-cell dependent pathway, the same as PWM. It might be assumed that the acetate group in eugenyl acetate did not alter the pattern of the B-cell proliferative response to eugenol. With PHA, eugenol activated splenocyte proliferation by approximately 34%, while eugenyl acetate had no effect. When the cells were exposed to either agent together with Con A, all concentrations of eugenol induced considerable stimulation of splenocyte proliferation which was 40% higher than that induced by eugenyl acetate. The results suggest that eugenol mainly stimulated T-cell proliferation, likely by indirect cross-linking of the T cell receptor, the same as Con A (Benjamini *et al.*, 2000). These findings might suggest that the acetate group in eugenyl acetate disturbed the T-cell proliferation pattern induced by eugenol. Previous studies have reported flavonoids affecting T-cell proliferation either to a greater extent than B-cell proliferation or affecting the two populations equally (Hirano *et al.*, 1989; Mookerjee *et al.*, 1986; Namgoong *et al.*, 1993; You *et al.*, 1998). This can be viewed to be in line with the differential effects of eugenol and eugenyl acetate, two

closely related phenolic compounds, seen on T- and B-cell proliferation in our results.

Fully differentiated T helper (Th) lymphocytes are divided into at least two distinct subsets based on cytokine production. Th1 cells are involved in cell-mediated immunity and produce cytokines such as IFN-γ and IL-2. These cytokines serve to activate monocytes/macrophages, natural killer cells, and cytotoxic T cells and they are associated with the host defense against bacteria, viruses, and fungi (Mosmann & Sad, 1996). In contrast, Th2 cells are involved in humoral immunity and produce cytokines such as IL-4 and IL-10 which are associated with the allergic response (Romagnani, 2000). Moreover, modulation of cytokine release by immunomodulating agents is an attractive target for the treatment of several pathological conditions such as infection, allergy, autoimmune diseases, and cancer (Elenkov & Chrousos, 1999; Mu *et al*., 2000; Tzianabos, 2000).

Viewed according to their maximal activities, both eugenol and eugenyl acetate affected the secretion of Th1 (IFN-γ and IL-2) and Th2 (IL-10) cytokines similarly. They markedly suppressed IFN-γ secretion, moderately inhibited IL-2 production, and increased IL-10 production dramatically in a dose dependent manner. Nevertheless, eugenol was more potent than eugenyl acetate in activating Th1 cytokine secretion while eugenyl acetate was more potent than eugenol in activating Th2 cytokine secretion, since the effective concentration was observed at lower doses. These results suggest that both eugenol and eugenyl acetate have anti-inflammatory actions on both Th1- and Th2-cytokine secretion. The acetate group in eugenyl acetate affected cytokine secretion from T-lymphocytes in response to eugenol only slightly. This was in accordance with the results with eugenol and eugenyl acetate in the splenocyte proliferation assay. The stimulation of Th2 cells led to an increment in IL-10, which effected the inhibition of Th1 cell proliferation and suppression of IFN-γ and IL-2, consecutively. This is supported by a previously reported down regulation of Th1 cytokines (TNF-α, IL-2) noted in nicotine treated macrophages with concurrent activation of a Th2 (IL-10, TGF-β) response (Mahapatra *et al*., 2011). However, the exact mechanism needs to be studied further. Moreover, the anti-inflammatory activity of eugenol derived from the cortex of *Eugenia caryophyllata* on the inhibition of prostaglandin E2 in LPS-stimulated RAW264.7 cells was reported. The mechanism of action was due to the suppression of cyclooxygenase-2 (COX-2) gene expression and direct inhibition of COX-2 enzyme activity. Eugenol also inhibited cell growth and suppressed COX-2 gene expression in human colon cancer cells which suggested the potential use as a cancer chemopreventive agent (Kim *et al*., 2003). A previous report used eugenyl acetate as an agent to inhibit and/or prevent the growth of and/or kill microorganisms that cause bad breath and/or to combat bad breath (Rabenhorst *et al*., 2006). Interestingly, our observation that eugenyl acetate has anti-inflammatory activity may also support its application in dental treatment.

Eugenol and eugenyl acetate are approved by the Food and Drug Administration as generally recognized as safe for food use. They are also used as a fragrance in cosmetics. To minimize the potent oxidative activity of eugenol, various esters of eugenol related compounds such as eugenyl acetate was synthesized (Chiaradia *et al*, 2012). Eugenol is a mild dermal irritant. However, oral eugenol can cause hepatotoxicity. Eugenyl acetate appears to be a non-irritant and non-allergenic substance, and possesses minimal acute and subchronic toxicity (Tisserand & Young, 2014). However, undiluted eugenyl acetate was moderately irritating to rabbit skin (Opdyke, 1979). Nanoparticulate systems might be applied to provide the vectorization of eugenyl acetate to specific targets and decrease the toxicity due to the lower concentration required for the therapeutic effect.

It might be concluded that eugenol and eugenyl acetate possess anti-inflammatory activities with different actions on different types of immune cells and should be useful in the application of anti-inflammatory therapeutic approaches. The acetate group in eugenyl acetate altered the immunological response pattern and eugenyl acetate may be useful either as a substitute or combined with eugenol to decrease the chances of high dose toxicity. Other assays are being carried out in order to permit a better understanding of its mechanism of action for further applications in the treatment of human immune mediated diseases.

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