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

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<th>Journal:</th>
<th>Songklanakarin Journal of Science and Technology</th>
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<td>Manuscript ID</td>
<td>SJST-2015-0083.R4</td>
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<tr>
<td>Manuscript Type:</td>
<td>Original Article</td>
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<tr>
<td>Date Submitted by the Author:</td>
<td>29-Jan-2018</td>
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<td>Complete List of Authors:</td>
<td>Saraphanchotiwitthaya, Aurasorn; Faculty of Pharmaceutical Sciences, Naresuan University, Department of Pharmaceutical Technology Khorana, Nantaka; Faculty of Pharmaceutical Sciences, Naresuan University, Department of Pharmaceutical Chemistry and Pharmacognosy Sripalakit, Pattana; Faculty of Pharmaceutical Sciences, Naresuan University, Department of Pharmaceutical Chemistry and Pharmacognosy</td>
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<td>Keyword:</td>
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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 was considered for substitution. The immunomodulatory activity of eugenyl acetate comparing 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 eugenol acetate, through the same pathway as concanavalin A. Moreover, both eugenol and eugenyl acetate showed similar maximal activity in inhibiting IFN-γ and 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 an alternative therapy for treatment of diseases is increasing. There are various investigations using 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, nutmeg (Raghavenra et al., 2006) and the major component (80–95%) of
clove oil (Szabolics & Erdelyi, 2000). It is widely used as a flavouring agent in food
products, 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, including 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-tumour activities, and it is also
used as local anaesthetic (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 (Shimoda et al., 2006;
Mastelić et al., 2008), which may cause high dose toxicity (Markowitz et al., 1992). At
concentration of higher than 3 mM, eugenol was cytotoxic to oral mucosal fibroblasts,
and decreased cellular ATP. Acute oral LD₅₀ was 2.13 g/kg in guinea pigs, 2.68 g/kg in
rats and 3.0 g/kg in mice, respectively (Tisserand and Young, 2014). The chemical
properties of eugenol and eugenyl acetate were studied by using DFT and HF methods.
It had been found that eugenyl acetate was more stable that eugenol while eugenol could
give reaction to radical forms; preventing the radical damage in cancer cells (Gökalp,
2016). Moreover, both clove oil and its esterified product, eugenyl acetate, presented
antimicrobial activities against gram-negative bacteria. They had high antioxidant
potential, particularly for eugenyl acetate (Vanin et al., 2014). Therefore, eugenol
derivatives such as eugenyl acetate have been considered in the study. Eugenyl acetate can be extracted from many spicy herbs just like eugenol (Jirovetz et al., 2006; Govinden-Soulan, et al., 2004) and has also been successfully synthesized by a 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 (Symons & King, 2003; Davicino et al., 2008). 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 has been investigated. An aqueous extract of clove inhibited macrophage production of IL1-β 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 oedema 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 leukaemia 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 (Lee & Shibamoto, 2001; Ito et al., 2005). Nevertheless, studies of the immunomodulatory activity of eugenyl acetate have been limited. Thus,
we study 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 from Fisher Scientific (Loughborough, UK), fetal bovine serum (FBS) and RPMI-1640 medium were purchased from GIBCO/BRL Invitrogen (Paisley, Scotland). IFN-γ, IL-2 and IL-10 ELISA kits were 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 µM 2-mercaptoethanol and 1 mL antibiotic-antimycotic solution). The exudates from 3 mice were pooled and centrifuged at 300 g, 25°C for 20 min and the cells were washed twice and re-suspended in RPMI medium. The purification of macrophages was performed using their ability to adhere to tissue culture plastic surface by culturing for 2 hr at 37°C. After that, non-adherent cells were removed by gently washing 3 times with RPMI. The adherent cells were rinsed vigorously with cRPMI and approximately 90-95% of macrophages as determined by morphologic criteria under microscope were obtained. The cell number was adjusted to 1×10^6 cell/mL as determined by counting in a haemocytometer. 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 spleens were removed aseptically. Cell suspensions were prepared by mincing and tapping the spleen
fragments on a stainless 200-mesh in RPMI medium (Manosroi et al., 2003). Splenocytes from 2-3 mice were pooled. After centrifugation at 300 g, 37°C for 10 min, the cells were washed twice and re-suspended in complete RPMI medium. The cell number was adjusted to 5×10^7 cell/mL by counting in a haemocytometer. 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% CO₂ 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% CO₂ 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 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% CO₂ 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 was added and the absorbance at 570 nm was measured by a microplate reader. The stimulation index (SI) was calculated as the ratio of the OD values of the sample and mitogen.

### 2.7 LPS-induced cytokine production assay

Production of mouse IFN-γ, IL-2 and IL-10 was 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% CO₂ incubator and the culture supernatants were analysed. 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 were expressed as mean ± S.E. Statistical differences (significance level $P < 0.05$) between groups were assessed using a one-way analysis of variance followed by multiple comparisons using Turkey’s method.

3. Results

The effects of eugenol and eugenyl acetate on the phagocytic activity of mouse macrophage were investigated using NBT dye reduction and lysosomal enzyme activity assays. Eugenol and eugenyl acetate were not toxic to macrophages at the tested concentrations because cell viability was greater than 90%. The results indicated that eugenyl acetate at 1-1000 $\mu$g/mL stimulated NBT dye reduction in a non-dose dependent manner, reaching a maximum stimulation of about 124% (SI; 2.2) at 10 $\mu$g/mL, while eugenol did not induce any change. Both eugenol (100 and 1000 $\mu$g/mL) and eugenyl acetate (1-1000 $\mu$g/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 that induced by 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 by LPS, and by 20–30% (SI; 0.7–0.8) when induced with PWM, 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, 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 flavouring agent in cosmetics and food products (Chang et al., 2002; Tai et al., 2002). Various pharmacological activities of eugenol have been investigated (Sen et al., 1992; Ueda-Nakamura et al., 2006; Park et al.,...
Due to aninstability issue with eugenol, eugenol derivatives such as eugenyl acetate have been considered for substitution. Eugenyl acetate is usually extracted from the same sources as eugenol but to a lesser extent (1–4%) (Jirovetz et al., 2006; Govinden-Soulange et al., 2004). 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 defence mechanisms against host infection and the killing of tumour 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 (Symons & King, 2003; Davicino et al., 2008). In the present studies, 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, reflecting stimulation of phagocytosis in proportion to intracellular killing (Rainard, 1986). 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 might be due to eugenyl acetate at high concentration might disturb macrophage function on oxidase enzyme production through various mechanisms (Maraldi, 2013; Lim et al., 2007). 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 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 effect 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 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
coloured 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, suggesting weak inhibition of acid
phosphatase production. Previous investigations have demonstrated that eugenol
induced ROS, leading to apoptosis in human promyelocytic (HL-60) leukaemia 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 phagocytosis beyond that induced by eugenol in the present study, it can be
assumed that 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 (Nakamura et al., 1986; Bekeredjian-Ding et al., 2012).

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 suggests 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-cells’ proliferative response to eugenol. With PHA, eugenol activated splenocyte proliferation 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, 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 (TCR), 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 (Mookerjee et al., 1986; Hirano et al., 1989; Namgoong et al., 1993; You et al., 1998). This can be viewed as 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 are
associated with the host defence 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, 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, anti-inflammatory activity of
eugenol derived from the cortex of Eugenia caryophyllata on the inhibition of
Prostaglandin E2 (PGE2) 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 directly inhibition of COX-2 enzyme activity. Eugenol also inhibited
cell growth and suppressed COX-2 gene expression in human colon cancer cells,
suggesting a potential use as cancer chemopreventive agents (Kim et al., 2003). There
was a previous report using eugenol 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 FDA as GRAS for food use.
They are also used as fragrance and flavoring agents 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
non-irritant and non-allergenic, and possesses minimal acute and subchronic toxicity
(Tisserand and Young, 2014). However, undiluted eugenyl acetate was moderately
irritating to rabbit skin (Opdyke, 1979). Nanoparticulated systems might be applied for
providing the vectorization of eugenyl acetate to specific targets and decreasing the
toxicity due to the lower concentration required for the therapeutical 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 eugenol 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 and to further apply it for the treatment of human immune mediated diseases.

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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 comparing to control, *; P < 0.05.
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 comparing 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 comparing to LPS, *; P < 0.05.