Eugenol and Its Role in Chronic Diseases

S. Fujisawa and Y. Murakami

Abstract The active components in cloves are eugenol and isoeugenol. Eugenol has recently become a focus of interest because of its potential role in alleviating and preventing chronic diseases such as cancer, inflammatory reactions, and other conditions. The radical-scavenging and anti-inflammatory activities of eugenol have been shown to modulate chronic diseases in vitro and in vivo, but in humans, the therapeutic use of eugenol still remains to be explored. Based on a review of the recent literature, the antioxidant, anti-proliferative, and anti-inflammatory activities of eugenol and its related compounds are discussed in relation to experimentally determined antioxidant activity (stoichiometric factor *n* and inhibition rate constant) and theoretical parameters [phenolic O–H bond dissociation enthalpy (BDE), ionization potential (IP according to Koopman's theorem), and electrophilicity (ω)], calculated using a density functional theory method. Dimers of eugenol and its related compounds showed large antioxidant activities and high ω values and also exerted efficient anti-inflammatory activities. Eugenol appears to possess multiple antioxidant activities (dimerization, recycling, and chelating effect) in one molecule, thus having the potential to alleviate and prevent chronic diseases.

Keywords Eugenol · Antioxidant activity · Theoretical parameter · Anti-inflammatory activity · Preventing chronic diseases

1 Introduction

Cloves are an important spice with a wide range of traditional uses in non-Western countries, mainly as a medicinal antiseptic, analgesic, and antimicrobial agent [1, 2]. The main component of cloves is eugenol, and its isomer—isoeugenol—is produced

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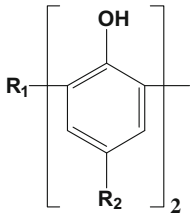
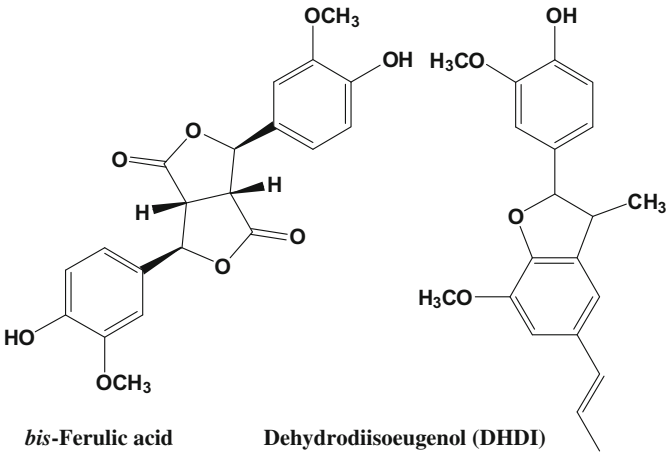
from eugenol via a reaction that occurs naturally in cloves. These compounds are incorporated into a variety of dental materials and household and personal hygiene products including perfumes, cream lotions, soaps, and detergents and are used as flavoring agents in non-alcoholic drinks, baked foods, and chewing gum [1]. Eugenol and its related compounds are effective antioxidants that can prevent free radical-mediated diseases such as cancers, inflammatory conditions, type-2 diabetes mellitus (DM), cardiovascular disease, neurodegenerative disorders, and periodontal disease [2–4]. They can act as free radical scavengers or generators, depending on their nature and concentration, and this dual effect may influence cell viability and anti-inflammatory activity to various degrees [5]. Investigations in our laboratory have focused on the antioxidant, anti-proliferative, and anti-inflammatory activities of eugenol and its related compounds, particularly dimers of eugenol and isoeugenol. Various dimers have been previously synthesized from 4-allyl-2-methoxyphenol (eugenol), 4-hydroxy-3-methoxy-1-propenylbenzene (isoeugenol), 2-*t*-butyl-4-methoxyphenol (BHA), 2-methoxy-4-methylphenol (MMP), and 4-hydroxy-3-methoxycinnamic acid (ferulic acid) monomers; and eugenol-dimer, dehydrodi-isoeugenol (DHDI), α-diisoeugenol (R-1-ethyl-5-hydroxy-*t*-3-(4-hydroxy-3-methoxy phenyl)-6-methoxy-c-2-methylindane), MMP-dimer, BHA-dimer, and ferulic acid-dimer (*bis*-ferulic acid) (Fig. 1) [3, 6–9]. The antioxidant, anti-proliferative, and anti-inflammatory activities of these compounds together with curcumin, tetrahydrocurcumin (THC), magnolol, honokiol, 2,2′-biphenol, 4,4′-biphenol, etc., have also been investigated [10–24]. Their antioxidant activity was determined using the induction period method developed in our laboratory [6–9] and also the well-known 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The anti-proliferative activity was determined by the [3-(4,5-di-methylthazol-2-yl)-2,5-diphenyltetrazolium bromide, yellow tetrazole (MTT)] assay, and the anti-inflammatory activity by the assessment of the inhibitory effects of cyclooxygenase (Cox)-2 and/or nuclear factor kappa B (NF-κB) on lipopolysaccharide (LPS)- or *Porphylomonas gingivalis* (Pg)-fimbria-stimulated RAW264.7 cells (a murine macrophage-like cell line) using northern blotting, Western blotting, and other techniques [10–24]. Here, we present our results and discuss the antioxidant, anti-proliferative, and anti-inflammatory activities of eugenol and its related compounds, which are of significance for selecting and designing novel nonsteroidal anti-inflammatory drug (NSAID)-like phenolic compounds for the treatment of chronic diseases. Also, the role of eugenol in the prevention of chronic diseases is discussed in relation to experimentally determined antioxidant activity [stoichiometric factor n and inhibition rate constants (k_{inh}/k_p)] and theoretical parameters (BDE, IP, and ω), which were calculated using a density functional theory (DFT) method [3, 9, 14, 19–24]. The biological activity of eugenol in animals and humans was also investigated by a review of the literature.



dimer	R_1	R_2
Eugenol	Methoxy	Allyl
MMP	Methoxy	Methyl
BHA	2-*t*-Butyl	Methoxy
Magnolol	H	Allyl
2,2'-Biphenol	H	H



***bis*-Ferulic acid** **Dehydrodiisoeugenol (DHDI)**

Fig. 1 Structure of dimers

2 Physicochemical Properties of Eugenol

2.1 Chemistry and Metabolites

Eugenol is generally well soluble in organic solvents and sparingly soluble in water (log $P = 2.49$). Since the environments inside most living organisms are heterogeneous, a certain degree of hydrophobicity is necessary in order for antioxidants to penetrate cellular membranes. Eugenol lowers the phase transition temperature and decreases the enthalpy (ΔH) of L-α-dipalmitoylphosphatidylcholine (DPPC) liposomes in a biomembrane model and also shows little diffusion from eugenol/DPPC

liposomes (1:4 molar ratio) due to its strong hydrophobic interaction with DPPC, as determined by differential scanning calorimetry (DSC) and NMR studies, respectively [25, 26]. This indicates that eugenol directly penetrates the lipid bilayer of liposomes, and exists on the surface. Eugenol is able to prevent free radical-mediated lipid peroxidation in cellular membranes containing unsaturated fatty acids by acting as an antioxidant. Indeed, eugenol has been shown to inhibit non-enzymatic peroxidation in liver mitochondria [27]. Also, eugenol binds to proteins such as serum albumin through hydrophobic interaction [28]. On the other hand, eugenol can also act as a prooxidant. Under alkaline conditions at pH 9.5, eugenol produces a phenoxyl radical at room temperature with a half-life of about 3.5 min, as determined by electron spin resonance spectroscopy (ESR), suggesting that the eugenol phenoxyl radical can exist for relatively long time in cellular systems [6]. Eugenol is converted to eugenol quinone methide (QM) via the one-electron oxidation pathway, and eugenol-QM intermediates bind to thiols such as glutathione (GSH). In the formation of oxidized GSH, oxygen consumption is increased, and a thiyl radical becomes detectable. GSH then reacts with the eugenol-QM, resulting in the formation of a eugenol-GSH conjugate [29]. The metabolism of eugenol leads to the production of cytotoxic compounds, particularly involving two pathways: a peroxidation reaction and a reaction catalyzed by P-450 microsomal enzymes [29]. This may lead to production of the 2′,3′-oxide of eugenol. Another study has revealed that the anti-DPPH radical activity of eugenol shows slow kinetics, whereas that of isoeugenol shows rapid kinetics [30]. This suggests that eugenol produces a phenoxyl radical, whereas isoeugenol produces a benzyl radical [13]. In humans, eugenol is rapidly absorbed and metabolized after oral administration, and almost completely excreted into urine as 4-hydroxy-3-methoxyphenyl-propane, isoeugenol, and other compounds [31].

2.2 *Antioxidant Activity (Stoichiometric Factor* N *and* K_{inh}/K_p*)*

Antioxidants have two forms [32]: peroxide-decomposing (preventive) antioxidants and conventional chain-breaking antioxidants. In biological systems, a variety of enzymes (such as superoxide dismutase (SOD), catalase, GSH peroxidase, GSH reductase), scavenge reactive oxygen species (ROS, comprising the hydroxyl radical OH^{-}, nitric oxide NO, the peroxy radical $ROO^{\cdot}$, and the alkyl radical $R^{\cdot}$), and mediate cellular events such as induction of apoptosis and necrosis. Biological systems can generate high amounts of ROS following an oxidant challenge, such as the presence of LPS, bacterial fimbriae, pro-inflammatory cytokines, or chemical and physical factors [3, 4]. Enzymes such as antioxidants prevent the generation of radicals indirectly, whereas eugenol-related compounds scavenge the radicals directly and are known as chain-breaking antioxidants. In general, the reproducibility of lipid peroxidation by free radicals in biological systems is poor

because of auto-oxidation initiated by minute and highly variable quantities of impurities (e.g., peroxide and transition metal ions). Therefore, some kinetic studies have examined the use of $ROO^{\cdot}$ or $R^{\cdot}$ radicals. The induction period (IP_t) method has been generally applied for evaluating the inhibition rate constant (k_{inh}) of various phenols and amines in the chlorobenzene/styrene-azoinitiator system [32] and also in linoleic acid/sodium dodecyl sulfate micelles initiated by the water-soluble azoinitiator system under O_2 at 760 torr [33]. However, these studies were carried out in air, and some compounds were not detectable because their indicated induction period was too small to measure using this system [33]. By contrast, we have previously proposed the use of DSC and the induction period method in a methyl methacrylate (MMA)–benzoyl peroxide (BPO) system under nearly anaerobic conditions. This IP_t method has proved to be reliable for evaluating the activity of phenolic compounds because the DSC technique is very sensitive and extraordinarily precise [3, 6–9]. Also, living organisms have a low oxygen tension (15 torr) [32] and cancer cells are well known to exhibit anaerobic metabolism (i.e., they do not utilize oxygen). Although such initiators employed in chemical studies are not present in biological systems, data obtained in kinetic studies are useful for interpreting the mechanisms of free radical-mediated biological activities. The antioxidant activities of 23 eugenol-related compounds are shown in Table 1.

The n value (the number of free radicals trapped by one mole of phenolic antioxidant moiety) is calculated from the IP_t in the presence of inhibitors [IH] as follows:

$$n = (R_i \times [IP_t])/[IH] \quad (1)$$

where R_i is the rate of initiator BPO decomposition at 70 °C, i.e., 2.28×10^{-6} mol l^{-1} s^{-1} in this work [8, 9].

k_{inh}/k_p can be calculated using the following equation:

$$k_{inh}/k_p = [MMA]/([IP_t] \times [\,Rp_{inh}]) \quad (2)$$

where Rp_{inh} is the rate constant for chain propagation in the presence of an inhibitor. [MMA] is the concentration of methyl methacrylate and is 9.4 mol/l.

The n value of 10 monophenols and 2 polyphenols (hesperatin and hesperidin) declined in the following order: 4-hydroxyanisole (2.4) > BHA (2.2) > 2,6-di-*t*-butyl-4-methoxyphenol, DTBMP (2.00 as a control) > isoeugenol (1.7) > ferulic acid ≈ MMP (1.6) > eugenol (1.4) > guaiacol (1.1) > hesperetin (0.9) > DHDI (0.8) > vanillin (0.2) > hesperidin (0.04). In general, a monofunctional phenol reacts with two $ROO^{\cdot}$ radicals to give a product that is stable, giving an n of 2. If the products themselves become inhibitors, this monophenol would lead to higher n values ($n > 2$), which would vary according to the nature of the second reaction, and this was the case for BHA and 4-hydroxy anisole with substituted OCH_3 at the *para* position. By contrast, the n value of eugenol, ferulic acid, MMP, and isoeugenol was reduced to 1.3–1.7 and that of guaiacol, DHDI, and hesperetin to

Table 1 Antioxidant activities and theoretical parameters (IP and BDE) for eugenol-related compounds

ID	Compound, CAS No.	IPM		Anti-DPPH·	IP	BDE
		n[a]	k_{inh}/k_p[b]	IC_{50} (mM)[c]	eV	kJ mol^{-1}
	(A) Guaiacol group					
1	Eugenol, 97-53-0	1.4	7.07	0.082	5.45	346.8
2	Eugenol-dimer, 4433-08-3	2.3	6.71	0.042	5.19 (5.46)	336.5 (354.0)
3	Isoeugenol, 97-54-1	1.7	9.0	0.055	5.18	339.2
4	α-diisoeugenol	2.7	5.87	0.05	5.28 (5.30)	343.2 (347.2)
5	DHDI, 2680-81-1	0.8	18.29	1.3	5.2	–
6	Vanillin, 21-33-5	0.2	101.1	27.4	6.08	361.9
7	Guaiacol, 90-05-1	1.1	15.71	0.108	5.53	364.6
8	MMP, 93-51-6	1.6	8.96	0.09	5.38	344.1
9	MMP-dimer	2.4	6.26	0.02	5.11 (5.02)	338.1 (338.2)
10	Curcumin, 458-37-7	3.9	4.89	0.043	5.27 (5.37)	344.0 (347.0)
11	THC, 36062-04-1	3.2	5.04	0.035	–	–
12	Ferulic acid, 537-98-4	1.6	10.56	0.145	5.7	355.7
13	*bis*-Ferulic acid	–	–	3.16[d]	5.77 (5.89)[d]	360.2 (360.9)[d]
14	Hesperetin, 520-33-2	0.9	18	–	–	–
15	Hesperidin, 520-26-3	0.02	362	–	–	–
	(B) Biphenol group					
16	Honokiol, 35354-74-6	3.2	4.77	–	5.87 (5.53)	396.4 (403.3)
17	Magnolol, 528-43-8	2.2	6.71[d]	–	5.51 (5.47)	392.4 (405.1)
18	2,2′-biphenol, 1806-29-7	0.8	32.19	–	5.97 (5.67)	287.3 (449.1)
19	4,4′-biphenol, 92-88-6	2.4	10.21	–	5.35 (5.44)	346.1 (346.1)
	(C) Anisole group					
20	4-hydroxyanisole, 150-76-5	2.4	8.16	–	5.35	343.6[e]
21	BHA, 121-00-6	2.15	6.53[d]	–	5.3	325
22	BHA-dimer	2.1	6.65	0.012	5.38 (5.15)	312.8 (319.7)
23	DTBMP, 489-01-0	(2.00)	8.21[d]	0.09	–	327.3[e]

IPM Induction period method; *IP* Ionization potential according to Koopman's theorem; *BDE* Phenolic O–H bond dissociation enthalpy

[a]Stoichiometric factor n: the number of free radicals trapped by one mole of phenolic antioxidant moiety

[b]The ratio of the rate constant of inhibition to propagation, k_{inh}/k_p

[c]The amount of inhibition necessary to decrease to the initial DPPH concentration by 50 %, DPPH 0.1 mM

[d]This work

[e]Taken from [37]; ( ), 2nd oxidation value (see the text)

Note that IDs 1–10 and 21–22 were taken from [8, 9]. IDs 11, 12, and 20 were taken from [14, 21, 24], respectively. IDs 16 and 17 from [23]. IDs 18 and 19 from [22]

approx. 1. Such a reduction is probably due to the strong hydrogen bond between the phenolic O–H and OCH_3 substituents on the benzene ring, and compounds having an n value of less than 2 would undergo dimerization due to the *ortho–ortho* coupling reactions derived from antioxidant phenoxyl radicals [30, 34]. The free radical coupling reaction of the guaiacol non-enzymatically leads to the formation of dimeric intermediates [35]. For the n values of the guaiacol group see Table 1. By contrast, the n value for vanillin, a guaiacol group, was 0.2, making it a very weak antioxidant, which may be explained by the presence of electron-withdrawing CHO substituent at the *para* position. For phenol dimers, the n values of curcumin, THC, honokiol, and α-diisoeugenol were 3–4 and they were strong antioxidants. In contrast, the n values of 4,4′-biphenol and magnolol and the dimers of eugenol, BHA, and MMP were 2.1–2.4 and these compounds were weak antioxidants. Interestingly, the n value of 2,2′-biphenol, a stereoisomer of 4,4′-biphenol, was about 1, suggesting the formation of a dimeric compound from 2,2′-biphenol molecules. Note that the n value of fully oxidized phenol dimers should be 4.

The anti-DPPH$^{\cdot}$ radical activity of eugenol-related compounds is also shown in Table 1. The activity of vanillin was poor. The IC_{50} value of DHDI and *bis*-ferulic acid was 1.3 and 3.2 mM, respectively, indicating that these compounds were considerably weak antioxidants. By contrast, the IC_{50} values for curcumin, THC, α-diisoeugenol, eugenol-dimer, MMP-dimer, and BHA-dimer were 0.02–0.05 mM, their antioxidant activity being higher than that of DHDI, *bis*-ferulic acid, or the corresponding monophenols.

The k_{inh}/k_p values for a series of 23 selected eugenol-related compounds determined by the IP_t are also shown in Table 1. Hesperidin and hesperetin are polyphenols, but are grouped as monophenols because they have one hydroxyl substituent in the B ring. The k_{inh}/k_p values of monophenols declined in the following order: hesperidin (362) > vanillin (101) > DHDI (18) ≈ hesperetin (18) > guaiacol (16) > ferulic acid (11) > MMP ≈ isoeugenol (9) > DTBMP ≈ 4-hydroxyanisole (8) > BHA (7) ≈ eugenol (7). By contrast, the k_{inh}/k_p values of the dimers declined in the order 2,2′-biphenol (32) > 4,4′-biphenol (10) > magnolol ≈ eugenol-dimer ≈ BHA-dimer (7) > MMP-dimer ≈ α-diisoeugenol (6) > THC ≈ honokiol ≈ curcumin (5). For monophenols, the k_{inh}/k_p values for eugenol and BHA were the smallest, followed by 4-hydroxy anisole. BHA and 4-hydroxy anisole are well known to be polymerization inhibitors. Eugenol and BHA were the most efficient radical scavengers. Among the dimers, honokiol, THC, and curcumin were the most potent antioxidants. In the present study, there was a good significant relationship between anti-DPPH radical activity (IC_{50}) and the k_{inh}/k_p value for compounds classified as guaiacols (n = 15, $r^2 = 0.98$, $p < 0.01$); the IC_{50} value increased along with the k_{inh}/k_p value. However, there was a weak relationship between the IC_{50} and the stoichiometric factor n value ($n = 15$, $r^2 = 0.30$, $p < 0.05$). Considering the effectiveness of the n and k_{inh}/k_p values, the n value may be useful for estimation of intermediates and by-products produced during the IP_t.

To clarify the co-oxidation mechanism of thiols, the radical-scavenging activity of eugenol, isoeugenol, and curcumin in the presence of mercaptomethylimidazole (MMI), a thiol, was investigated using the IP_t method in the BPO-MMA system.

MMI was used as a representative thiol, since attempts to use *N*-acetylcysteine (NAC) and GSH-bearing SH groups were unsuccessful because of the fact that NAC and GSH show only limited solubility in MMA. The IP_t for the combination of curcumin, isoeugenol, or eugenol with MMI was compared to that without MMI. The curcumin/MMI (1:1 molar ratio) and isoeugenol/MMI (1:1) complex, particularly the former, showed a decrease in IP_t, indicating an antagonistic effect between the antioxidant and MMI. Conversely, the eugenol/MMI complex (1:1) showed an increase in the IP_t, indicating a synergistic effect [7]. Therefore, it was assumed that MMI reacted with the eugenol phenoxyl radical and reduced it back to the parent eugenol compound. Such a synergistic (recycling) effect and the formation of eugenol conjugates have been reported previously [29].

2.3 *Theoretical Parameters (BDE and IP) Versus* K_{inh}/K_p

In recent years, theoretical methods in combination with physical organic chemistry theory have found broad applications in studies of antioxidants. Theoretical calculation can offer a deep insight into differences in radical-scavenging mechanisms and antioxidant activities among various phenolic compounds with a wide range of structures [36]. The BDE, the lowest unoccupied molecular orbital (LUMO) energy (E_{LUMO}), the highest occupied molecular orbital (HOMO) energy (E_{HOMO}), the IP value according to Koopman's theorem (absolute HOMO value), and the η, χ, and ω values for eugenol-related compounds were taken from our previous reports [3, 8, 9, 14, 19–24], and all calculations were performed using the density function theory (DFT)Becke-3-LYP (B3LYP)/ 6-31G* method [8, 9].

BDE is the most widely used parameter of radical-scavenging activity for phenolic antioxidants and is also correlated well with the logarithm of the inhibition (radical-scavenging) rate constant (k_{inh}) for chain-breaking antioxidants. From a kinetics viewpoint, the thermodynamically preferred mechanism accords with the following equation [36, 37]:

$$-RT \ln k_{inh} = \Delta G^0_{\#} \approx BDE \tag{3}$$

where $\Delta G^0_{\#}$ is the activation free energy. Equation 3 indicates a certain correlation between the k_{inh}/k_p and BDE values. Note that k_p is a propagation rate constant for MMA. The relationship between the k_{inh}/k_p and BDE values for 17 selected eugenol-related compounds was investigated. Except for magnolol and honokiol, a significant linear relationship in terms of BDE (note, however, that the BDE for dimers is BDE_{2nd}, a second H atom abstraction from the phenoxyl radical) was observed as follows:

$$\begin{aligned} \mathrm{Log}\, k_{\mathrm{inh}}/k_p &= -1.14(\pm 0.28) + 0.01\,(\pm 0.08)\,\mathrm{BDE} \\ &\left(n = 17,\ r^2 = 0.33,\ p < 0.05,\ p = 0.015\right) \end{aligned} \tag{4}$$

As the BDE increased, the k_{inh}/k_p also increased. Thus, the BDE of eugenol-related compounds probably plays a key role in the determination of antioxidant activity, reflecting the importance of hydrogen atom (H) transfer for radical scavenging.

Another report has demonstrated a good relationship between the k_{inh}/k_p and BDE or IP value of 2-methoxyphenols in each descriptor ($n = 5$, $r^2 = 0.95$, $p < 0.01$) [8]. Also, in the present study, a significant relationship between the k_{inh}/k_p and IP values of 23 eugenol-related compounds was observed, but the r^2 value was smaller than that of the BDE in Eq. (4). Although there was a significant relationship between the antioxidant activity and the BDE or IP value for eugenol-related compounds, the molecular mechanism regulating the antioxidant activity may be more complex than hydrogen (H)-atom abstraction, electron transfer, or proton transfer [38].

3 Modulation of Cell Signaling Pathways by Eugenol

Chronic inflammatory diseases are mediated by oxidative stress, which can activate a variety of transcription factors including NF-κB, activator protein-1 (AP-1), p53, hypoxia-inducible factor-1α (HIF-1α), peroxisome proliferator-activated receptor-γ (PPAR-γ), β-catenin/Wnt, and nuclear factor erythroid 2-related factor 2 (Nrf2) and other factors. Activation of these transcription factors can lead to the expression of over 500 different genes, including those of growth factors, inflammatory cytokines, chemokines, cell cycle regulatory molecules, and anti-inflammatory molecules [4]. The anti-proliferative and anti-inflammatory activities of eugenol and its related compounds are described in the following paragraphs.

3.1 Anti-proliferative Activity

A number of studies have investigated the mechanism responsible for the antioxidant activity of eugenol. In the HSG (human submandibular) cell line, the cytotoxicity of eugenol determined by the MTT method was one order of magnitude lower than that of isoeugenol (CC_{50}: eugenol, 0.395 mM; isoeugenol, 0.052 mM), and production of ROS (determined by carboxy-2′,7′ dichlorofluorescein diacetate (CDF) staining) was induced significantly by isoeugenol, but not by eugenol. In the presence of H_2O_2 plus horseradish peroxidase, or under visible light irradiation (which induces oxidative stress), eugenol triggered biphasic ROS production that was enhanced at lower concentrations (5–10 μM) and decreased at higher

concentrations (500 μM). In contrast, isoeugenol enhanced ROS production over a wide range of concentrations (5–500 μM). Isoeugenol at cytotoxic high concentrations of 1000 μM was reduced to below the detectable levels. The high cytotoxicity of isoeugenol may be attributed to its induction of high ROS production and low GSH levels [13]. The decrease in the ROS level at higher concentrations of eugenol may be responsible for its ROS scavenging activity. Indeed, eugenol scavenged hydroxyl radicals (OH^-) effectively and also trapped OH^- directly, as determined by the electron spin resonance (ESR) spectroscopy, and was subsequently metabolized to the dimer in vitro [39]. Thus, ROS at high concentrations may have been scavenged by eugenol, and therefore, cell viability was not altered. This action of eugenol appears to be greatly different from that of isoeugenol. In general, elevated levels of ROS lead to oxidation of proteins, lipids, and nucleic acids. ROS are the main products of cellular redox processes and exert a dual effect; a low concentration of ROS can be beneficial for cellular redox signaling and immune function, but a high concentration may result in oxidative stress and subsequent damage to cell function and structure [40]. As an antioxidant, eugenol may be a substance that can scavenge harmful free radicals such as ROS and help reduce the incidence of damage due to oxidative stress, thus helping to maintain cellular redox balance.

With regard to the kinetics of their antioxidant action, cloves, eugenol and isoeugenol, are known to produce dimeric compounds and other metabolites, which probably have cell-type specificity. It has been reported that the CC_{50} values of isoeugenol for HL-60 cells, human gingival fibroblasts (HGF), and human pulp cells (HPC) were 30, 32, and 37 μM, respectively, whereas those of eugenol were 178, 232, and 214 μM, respectively. By contrast, the corresponding values of eugenol-dimer were 105, 666, and 369 μM, respectively [8]. The cytotoxicity of isoeugenol for all cell types was one order greater than that of eugenol, and interestingly, eugenol-dimer was active on HL-60 cells. In another study, eugenol-dimer was more toxic than eugenol to HL-60 cells, and DNA fragmentation was induced most strongly by eugenol-dimer, followed in order by eugenol, MMP, and MMP-dimer. Furthermore, in HL-60 cells, the expression of mRNAs for manganese (Mn) SOD and copper (Cu)/zinc (Zn) SOD, particularly the latter, was inhibited by eugenol at 1 mM and the inhibition was strongly potentiated by the addition of GSH [11]; eugenol suppressed Cu/Zn SOD activity and increased the intracellular superoxide concentration, possibly acting as an inhibitor of cytosolic (Cu, Zn) at higher concentration through its action as a metal-ion chelator. In another study using HL-60 cells, eugenol treatment reduced the mitochondrial membrane potential and also resulted in reduction of bcl_2, release of cytochrome *c*, and activation of caspase-9 and caspase-3 [41]. On the other hand, some dimeric forms of eugenol (C2-symmetric structure, as shown in Fig. 1) showed anti-proliferative activity against melanoma cells, indicating that eugenol-dimer has mild activity, whereas curcumin and a racemic mixture of brominated biphenyl have potent activities. Some synthesized curcumin-related hydroxylated biphenyls have been reported to show higher anti-proliferative activities than curcumin itself [42]. In another study, eugenol at a concentration of 0.5 μM inhibited the growth of

SBC12 primary melanoma cells and VM3211 cells in radial growth phase by 50 %, whereas isoeugenol did not inhibit melanoma cell growth up to a concentration of 0.5 μM. Eugenol, but not isoeugenol, inhibits the proliferation of melanoma cells by arresting them in S-phase of the cell cycle and inducing apoptosis [43].

In another context, where interleukin (IL)-1β-stimulated HGF cells and periodontal ligament fibroblasts have been reported to produce large amounts of IL-8, treatment with eugenol stimulated the production of IL-8 [44]. In HPC cells, eugenol inhibited IL-8 production at a higher concentration [44]. Also, in HSC-2 cells (human oral squamous cancer cell), eugenol induced non-apoptotic cell death [45]. Taken together, these results imply that eugenol is probably capable of manipulating the equilibrium between pro- and anti-apoptotic proteins [46] and also has cell-type specificity.

There are some stereoisomers among eugenol-related compounds. The mechanism responsible for the cytotoxicity of isomers and stereoisomers is complex. In studies using RAW264.7 cells, magnolol, honokiol, 2,2′-biphenolm and 4,4′-biphenol were tested for their potential cytotoxicity, and the data indicated that magnolol was more cytotoxic than honokiol, and 4,4′-biphenol was more cytotoxic than 2,2′-biphenol [22, 23]. Magnolol and 2,2′-biphenol had lower IP values than the corresponding stereoisomer, and the lower IP value enhanced the cytotoxicity. A low IP may enhance prooxidant activity via direct transfer of an electron to oxygen [47].

Phenol-induced cytotoxicity is related to the phenoxyl radical, an oxygen-centered radical; this radical may interact with redox-sensitive cysteines in DNA-binding domains of transcription factors, or it may represent a slightly enhanced transport of the phenoxyl radical in a cellular environment. The strong correlation between the IP and BDE values suggests that phenol-induced cytotoxicity might be attributable to the radical-mediated mechanism [48]. A significant linear relationship between cytotoxicity (log $1/CC_{50}$) to HSG or HGF cells and the k_{inh}/k_p value was observed for both 2-methoxy- and 2-*t*-butyl-phenols ($n = 13$, $r^2 = 0.5$, $p < 0.01$) [9]. This suggests that the cytotoxicity of these compounds may also be related to the BDE value, resulting from Eq. (4). In general, compounds with higher BDE (or IP) values were less toxic. For phenolic compounds, electron-donating groups reduce their BDE and IP value, whereas electron-withdrawing groups have opposite effects [47]. These findings indicate that eugenol-induced cytotoxicities, toxicities, and anticancer activities are probably related to its intermediates, including antioxidant-derived radicals.

3.2 Cox-2 Inhibition

Cox-2 is a major contributor to increases in the spinal level of prostaglandin E_2, which augments the processing of nociceptive stimuli following inflammation. We therefore focused on the Cox-2-inhibitory activity of selected eugenol-related

compounds. The inhibitory effects of eugenol-related compounds in macrophage cell lines activated with LPS or Pg. fimbriae have been investigated in our laboratory in order to develop more effective chemopreventive agents and to elucidate their mechanism of action [10–24], and the results are shown in Table 2.

The 50 % inhibitory concentration of COX-2 (IC_{50}, μM) declined in the following order: eugenol (500 <) > MMP (308) > eugenol-dimer (287) > hesperetin (256) > hesperidin (254) > MMP-dimer (250) > guaiacol (205) > vanillin (125) > ferulic acid (52) > THC (24) > isoeugenol (23) > honokiol ≈ magnolol (20) > 4-hydroxyanisole (15) > *bis*-ferulic acid (10) > BHA-dimer (9.2) > 2,2′-biphenol (7) > curcumin (6) > DHDI (0.1). The Cox-2-inhibitory activity of DHDI was the highest, followed by curcumin. NF-κB is a signaling molecule acting upstream of Cox-2 expression and also regulates the production of pro-inflammatory cytokines such as IL-6, tumor necrosis factor (TNF)-α, and prostaglandin E_2. The inhibitory effect of eugenol-related compounds on NF-κB activation is also shown in Table 2.

Table 2 Anti-inflammatory activities of eugenol-related compounds

Compound	COX-2 IC_{50} value (stimulated RAW264.7 cell) (μM)	NF-κB inhibition (stimulated RAW264.7 cell) (μM)	References
Eugenol	>500	−(500)	[10, 11]
Eugenol-dimer	286.8	++(500)	[10, 11]
Isoeugenol	23.4	ne	This work
DHDI	0.1	++(0.1)	[16]
α-diisoeugenol	>10	ne	[16]
MMP	307.9	ne	[11]
MMP-dimer	250	ne	[11]
Hesperetin	256.2	ne	[15]
Hesperidin	254.1	ne	[15]
Ferulic acid	52.1	ne	[14, 19]
bis-Ferulic acid	9.8	++(10)	[14, 19]
Guaiacol	205	−(250)	[20]
Vanillin	125	++(250)	[20]
2,2′-biphenol	6.9	++(10)	[22]
4,4′-biphenol	>10	ne	[22]
4-hydroxyanisole	15.3	+(10)	[24]
BHA	>10	−(10)	[17, 18]
BHA-dimer	9.2	++(10)	[17, 18]
Curcumin	5.8	++(10)	This work
THC	23.6	+(20)	[21]
Magnolol	20.2	++(40)	[23]
Honokiol	20.4	++(40)	[23]

++, 75 % inhibition <; +, 50 % inhibition ≫; −, no inhibition; ne, no experiment

In drug screening, it is generally considered that if the concentration of drug required for 50 % inhibition of COX-2 (IC_{50}) is less than 3 μM, this compound can be regarded as a strong enzyme inhibitor. When carrying out screening based on the activation of the Cox-2 pathway by serum-free stimulation of the human lung cancer cell line, A549, the IC_{50} threshold for candidate compounds should be less than 10 μM [49]. As has already been reported, eugenol demonstrated slightly higher Cox-2-inhibitory activity when assayed at a concentration of 1000 μM [50]. In the present study, the IC_{50} value of Cox-2 inhibition by eugenol was >500 μM. The IC_{50} values of vanillin and hesperidin, with a lower antioxidant activity, were 125 and 260 μM, respectively. The values of eugenol-dimer, MMP, and MMP-dimer were 250–300 μM, respectively. The IC_{50} values of magnolol, honokiol, and THC were approx. 20 μM, and these compounds were placed in a moderate activity group. Screening of 20 different analogs of curcumin at 50 μM showed that the inhibitory activity of curcumin on TNF-induced NF-κB-dependent reporter gene expression was most potent, followed in order by eugenol and zingerone [51]. The most effective curcumin is a compound with an aromatic *o*-methoxy phenolic group, α, β-unsaturated β-diketo moiety, and a seven-carbon linker, which is a C4-symmetric guaiacol dimer. As descried above, magnolol and honokiol without an *o*-methoxy group, 4-allylphenol dimers, showed the moderate activity; however, 4-allylphenol did not show Cox-2 inhibition at a concentration of 50 μM [52]. In another report, the IC_{50} values of honokiol and magnolol were relatively smaller concentration of approx. 15 μM [53]. Interestingly, the anti-inflammatory activity of C2-symmetric dimers, eugenol-, MMP-, BHA-, 4-allylphenol, and phenol-dimer (Fig. 1) was higher than that for the corresponding monophenols. The C2-symmetric dimers, with structural conformation, enhanced Cox-2 inhibition as well as antioxidant activity. Molecules having two symmetric potential binding moieties bearing a flexible unit of suitable length and nature would enhance binding affinity providing higher activity than those lacking these elements. The Cox-2-inhibitory activity of ferulic acid was weak, and notably, its dimer (*bis*-ferulic acid) with two symmetric potential binding moieties (Fig. 1) showed potent activity. Some kind of bioactive dimeric compound of ferulic acid should be produced via intracellular radical oxidation, as estimated from the n value of 1.6 for ferulic acid. Also, ferulic acid has been reported to interfere with the biological pathways involved in apoptosis induced by oxidative stress and inflammation caused by misfolding and aggregation of the amyloid-β peptide (Aβ) [54]. These beneficial effects may be enhanced by the formation of bioactive dimeric compounds.

The inhibitory effect of DHPI, an isoeugenol-dimer on NF-κB activation, was the most potent, followed by curcumin, *bis*-ferulic acid, and BHA-dimer.

Quantum chemical calculation might provide a closer insight into the molecular mechanisms of anti-inflammatory activity. The electronegativity ($\chi = -(E_{HOMO} + E_{LUMO})/2$) and chemical hardness ($\eta = (E_{LUMO} - E_{HOMO})/2$) principle can be applied at the level of ligand-receptor binding in order to predict the genotoxicity and carcinogenicity of various chemicals [55]. The molecular χ is first equalized with that of the receptor, leading to selection of a molecular fragment with χ

complementary to that of the receptor, or adjustment of the receptor pocket to fit with the ligand χ. From these hypotheses, it is assumed that the Cox-2 enzyme and NF-κB proteins activated by pro-inflammatory stimuli such as LPS, ROS, and bacterial fimbriae may be controlled by the χ value of phenolic antioxidants such as biphenols and polyphenols. In a similar context, the ω value ($\omega = \chi^2/2\eta$) in particular has been used for electrophilic ranking of reactive compounds, as it seems to be related to both biological effects and the reactivity of unsaturated compounds with nucleophilic additions [56, 57]. Therefore, the ω value may be highly applicable for estimating the inhibitory effect of eugenol-related compounds on Cox-2 expression [3]. The ω value may be related to the anti-inflammatory activity of these compounds because χ and η are key indicators of the overall reactivity of the molecules.

For monophenols, the ω values for eugenol, isoeugenol, guaiacol, MMP, BHA, 4-allylphenol, and DHDI were 1.13, 1.66, 1.16, 1.12, 1.40, 0.95, and 1.64 eV, respectively, whereas those for eugenol-dimer, MMP-dimer, and BHA-dimer were 1.69, 1.62, and 2.15 eV, respectively, and those for curcumin, magnolol, honokiol, and 2,2′-biphenol were 4.65, 2.36, 2.45, and 2.10 eV, respectively [3, 9, 23]. Note that the ω values for monophenols lie within the one-electron oxidation pathway, whereas those for dimers lie within the two-electron oxidation pathway. Curcumin had the largest ω value, followed in order by magnolol, honokiol, BHA-dimer, and 2.2′-biphenol. Curcumin with the highest ω value showed efficient anti-inflammatory activity. However, despite the potent anti-inflammatory activity of DHDI, this compound did not have a high ω value, and therefore, the activity of DHDI may be attributable to other factors such as the formation of dimeric compounds. Further studies to clarify the molecular mechanism of DHDI will be necessary.

4 Role of Eugenol in Chronic Diseases

Extensive research has demonstrated the mechanism by which persistent oxidative stress can lead to chronic inflammation, which in turn could cause many chronic diseases such as cancer, type-2 DM, stroke, obesity, arthritis, and others. Oxidative stress is defined as a disturbance in the balance between the production of ROS and antioxidant defenses through elimination by protective mechanisms [4]. ROS play a central role both upstream and downstream of the NF-κB and TNF-α pathways, which are located at the center of the inflammatory response. Chronic diseases are radical-mediated, and eugenol and its related compounds scavenge free radicals and help reduce the incidence of oxidative stress-induced damage, thus preventing chronic inflammatory diseases. We have been actively searching for phytophenol antioxidants that might have preventive effects against chronic periodontal disease (PD) and other oral diseases, including cancers, in view of the possible link between such oral diseases and systemic diseases.

For this purpose, we have investigated the anti-inflammatory activity of eugenol and its related compounds in vitro using RAW264.7 cells stimulated with LPS or Pg. fimbriae. Chronic PD is induced by an inflammatory host immune response to Pg pathogenic bacteria. LPS and Pg fimbriae produce large amounts of ROS and damage both the gingival tissue and alveolar bone [58]. Eugenol at relatively high concentrations inhibited Cox-2 expression in RAW264.7 cells stimulated with LPS or Pg fimbriae, and some eugenol-related compounds exerted potent anti-inflammatory activity at relatively low concentrations (Table 2). In another study, eugenol dose-dependently inhibited the receptor activator of NF-κB ligand (RANKL)-induced formation of multinucleated osteoclasts and tartrate-resistant acid phosphatase (TRAP) activity in RAW264.7 macrophages [59]. The therapeutic role of eugenol for chronic inflammatory diseases will be discussed in the following sections.

5 Biological Activities of Eugenol in Animal Models

Studies to demonstrate the chemopreventive efficacy of eugenol against free radical-mediated chronic diseases in vivo have been limited [60]. Some anticancer studies using chemically induced tumor models have been reviewed. Skin tumors were initiated by the application of 7,12-dimethylbenzanthracene (DMBA) and promoted by 12-*o*-tetradecanoylphorbol-13-acetate (TPA). Initiation with DMBA led to significant upregulation of p53 expression with a concomitant increase in p21 (WAF1) levels in epidermal cells, indicating induction of DNA damage. However, pretreatment with eugenol led to overexpression of these genes, which probably helped stimulate apoptosis of the damaged cells. Eugenol inhibited the activation of NF-κB and markedly protected against chemically induced skin cancer [61].

Also, eugenol exhibited chemopreventive effects against *N*-methyl-N′-nitro-N-nitrosoguanidine (MNNG)-induced gastric carcinogenesis in Wistar rats, as determined by the analysis of markers of apoptosis, invasion, and angiogenesis. Administration of eugenol induced apoptosis via the mitochondrial pathway by modulating the Bcl-2 family proteins, Apaf-1, cytochrome *c*, and caspases, and by inhibiting invasion and angiogenesis, as evidenced by changes in the activities of their markers. Administration of eugenol significantly reduced the incidence of MNNG-induced gastric tumors by suppressing NF-κB activation and modulating the expression of NF-κB target genes that regulate cell proliferation and survival. Eugenol is an attractive candidate for the prevention of tumor progression [46]. Another report has indicated that thioacetamide (TA)-induced hepatic injury in adult Wistar rats was suppressed by eugenol. Eugenol pretreatment prevented liver injury by decreasing cytochrome P4502E1 (CYP2E1) activity, lipid peroxidation indices, protein oxidation, and inflammatory markers. Eugenol pretreatment prevented DNA strand breaks induced by TA. Increased expression of the Cox-2 gene induced by TA was also abolished by eugenol [62]. These studies demonstrated that eugenol inhibits the upstream signaling molecule, NF-κB and NF-κB-regulated genes, and markedly protects against chemically induced breast, skin, gastric, or

hepatic cancer, possibly by virtue of its anti-proliferative, anti-inflammatory, and antioxidant activities.

On the other hand, both transition metals and radicals are well known to play key roles in a number of chronic diseases. Many natural products such as, ascorbic acid, α-tocopherol, and GSH are also known to possess both metal-chelating and radical-scavenging properties. Eugenol can also bind the transition metal ion, Zn^{2+} [3]. Zn is an essential component of numerous proteins involved in defense against oxidative stress, and deficiency of Zn may enhance DNA damage via impairment of DNA repair mechanisms. Additionally, Zn has an impact on the immune system and possesses neuroprotective properties [63]. Zn insufficiency has been associated with vulnerability to development of many tumors, whereas conversely, Zn treatment can inhibit tumor development. The fact that eugenol efficiently inhibits chemically induced tumorigenesis in animal models may be related to its simultaneous metal-chelating and radical-scavenging properties. Intrinsically, eugenol with Zn may be incorporated into the cellular bilayer due to the high liphophilic activity of eugenol. Pretreatment and administration of eugenol may help to protect the cells, tissues, and organs from damage due to tumor invasion and angiogenesis.

It has been reported that in male C57BL/6 J mice with hyperglycemia induced by a high-fat diet (HFD), eugenol significantly inhibited glucagon-induced glucose production and enhanced adenosine monophosphate (AMP)-activated protein kinase (AMPK) phosphorylation in HepG2 cells and primary rat hepatocytes. In an animal study, plasma glucose and insulin levels of eugenol-treated mice were decreased by 31–63 % in comparison with HFD controls. Eugenol effectively ameliorates hyperglycemia through inhibition of hepatic gluconeogenesis by modulating the calcium calmodulin kinase kinase (CAMKK)-c-AMP-response element-binding protein (AMPK-CREB) signaling pathway [64].

It has been considered that dehydrodiisoeugenol (DHDI) may effectively ameliorate hyperglycemia [65]. Type-2 DM is caused by a combination of insulin resistance and pancreatic β cell insufficiency. One of the receptor targets for the treatment of Type-2 DM is peroxisome PPARγ, which is a master ligand-activated transcription factor belonging to the nuclear receptor family. One potent anti-DM drug is a high-affinity agonist of PPARγ [66], bearing hydrogen bond donor and acceptor groups for interacting with threonine (Thr) 473. Thus, Thr 473 might be a critical site of interaction between the PPARγ ligand-binding domains and its agonists. A molecular modeling study has shown that DHDI exerts anti-DM activity in vitro [65].

Eugenol is well known to have antimicrobial, antinociceptive, and antiviral activities [1] and is effective for prophylaxis and treatment of vaginal and oral candidiasis in immunosuppressed rats [67, 68]. Thus, eugenol exerts anti-tumorigenic, anti-hyperglycemic, or immunosuppressive activity against chronic diseases in animal models. However, no well-controlled clinical studies of eugenol in human patients with various chronic diseases have yet been performed.

6 Biological Activities of Eugenol in Humans

Eugenol in cloves has been used to prevent infection and reduce pain and was approved in monographs of the expert panel German Commission E published between 1983 and 1993. Eugenol is widely used as the liquid constituent of zinc oxide eugenol (ZOE) chelate cement and 2-ethoxybenzoic acid (EBA)-modified ZOE cement in dentistry. ZOE has been used for pulp capping, root canal filling, and as an impression and surgical pack material. ZOE has been used as standard cement for fillings in dental work [3]. Although early evidence suggests that ZOE has promise for use in dentistry, its use as an impression material and surgical pack material is limited due to its weak allergenicity [69]. Eugenol is generally non-allergenic, although in sensitized individuals it may cause a range of tissue reactions from low-grade local to systemic. Low concentrations of eugenol are well known to exert local anti-inflammatory, antiseptic, and anesthetic effects on dental pulp. A eugenol-containing zinc oxide gel has been used as an intra-pocket delivery system for treatment of periodontitis. The gel can release eugenol into the gingival pocket at a very low concentration to prevent bacterial infection [70]. Also, eugenol may have antibacterial effects that are beneficial for dental hygiene, being included in materials such as toothpastes and mouthwashes. Since isoeugenol has potent allergenicity, isoeugenol-related compounds were evaluated by patch testing in 2262 patients, demonstrating a high degree of concomitant reactivity [71]. Reduction of sensitization potency achieved by dimerization of isoeugenol may lead to development of safer cosmetic ingredients. In the guinea pig maximization test, isoeugenol, the dimer beta-O-4-dilignol, and another dimer DHDI were classified as having extreme, weak, and moderate allergenicity, respectively [72]. These dimers may be promising candidates for cosmetic ingredients with a low sensitization risk, but no clinical trials have yet been performed. By contrast, isoeugenol acetate is present in perfumes, aftershaves, etc., but may cause contact allergy in isoeugenol-sensitized individual [73]. Many eugenol-related compounds are allergenic themselves, but are activated in the skin (e.g., metabolically) or before skin contact (e.g., via air oxidation) to form skin sensitizers [74]. Although many studies have investigated the use of eugenol for preventing chronic diseases in in vitro and animal models, the therapeutic use of eugenol in humans still remains unexplored.

7 Conclusions

We have presented the results of our experiments to determine the antioxidant, anti-proliferative, and anti-inflammatory activities of eugenol and its related compounds and discussed the molecular basis of their action when used for the prevention of chronic diseases on the basis of experimental antioxidant parameters and theoretical parameters with reference to the recent literature. Eugenol and its related compounds prevent free radical-induced chronic diseases due to their efficient

antioxidant activities (as a chain-breaking antioxidant). Also, eugenol exerts anti-inflammatory activities in in vitro and animal models through suppression of pro-inflammatory cytokines such as NF-κB, TNF-α, and ILs. Eugenol and its related compounds, particularly their dimers, may have beneficial effects in the prevention of various chronic diseases.

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