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Comparative Study on the Antioxidant and Biological Activities of Carvacrol, Thymol, and Eugenol Derivatives

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Four derivatives of thymol, carvacrol, and eugenol were synthesized: 4-(hydroxymethyl)-5-isopropyl-2-methylphenol, 4,4′-methylenebis(5-isopropyl-2-methyl)phenol, 4-allyl-6-(hydroxymethyl)-2-methoxyphenol, and 4-(hydroxymethyl)-2-isopropyl-5-methylphenol. The obtained derivatives showed remarkably better antioxidative properties according to 1,1-diphenyl-2-picrylhydrazyl assay (50% inhibitory concentrations = 4–156 μg/mL) and Rancimat assay (protection factors = 1.55–5.84) when compared with parent compounds and values similar to or better than those of butylated hydroxytoluene and vitamin C. At concentrations of 10 mM carvacrol derivatives had no toxic effect on viability of *Escherichia coli* K-12 (determined by minimum inhibitory concentrations). Other phenol derivatives showed reduced cytotoxic effect on *E. coli* K-12 at concentrations of 2–5 mM on the basis of 50% lethal dose measurements. In comparison with the parent compounds, phenol derivatives showed reduced cytotoxic effect for *Saccharomyces cerevisiae* cells (determined by yeast colony reduction). On the other hand, the majority of synthesized compounds had dose-dependent antiproliferative effects on human uterine carcinoma cells (HeLa), which makes them potentially interesting for the adjuvant experimental cancer treatments. The 4,4′-methylenebis(5-isopropyl-2-methyl)phenol derivative of carvacrol showed lower inhibiting capacity also for the HeLa cells, which makes this particular derivative attractive as an efficient antioxidant with negligible cytotoxic effects.

KEYWORDS: Carvacrol; eugenol; thymol; hydroxymethyl derivatives; diarylmethane derivative; antioxidant activity; antiproliferative activity; antimicrobial activity; antiyeast activity

INTRODUCTION

Oxidative stress, caused by an imbalance between antioxidant systems and the production of oxidants, seems to be associated with many diseases, especially cancers, cardiovascular diseases, and inflammatory disorders. The mechanisms by which these pathologies develop generally involve oxidative alteration of physiologically critical molecules, including proteins, lipids, carbohydrates, and nucleic acids, along with modulation of gene expression and the inflammatory response (*1, 2*).

Autoxidiation of lipids has long been recognized as a major deterioration process affecting both the sensory and nutritional qualities of foods. The high oxidation stability of lipids can be ensured by the addition of antioxidants (*3*). The most common natural antioxidants are vitamin E (α-tocopherol), vitamin C, and various phenol compounds. These natural antioxidants can be found in fruits, vegetables, cereals grains, tea, oils, and many spices (*4–8*). Synthetic antioxidants such as 2,6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene, BHT) and 2- or 3-*tert*-butyl-4-methoxyphenol (butylated hydroxyanisole, BHA) are often added to food to prevent autoxidation. Phenols are able to donate H-atoms of phenol hydroxyl groups in reaction with peroxyl radicals that can produce stabilized phenoxyl radicals, thus terminating lipid peroxidation chain reactions. The

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antioxidant activity of phenols depends on the electronic and steric effects of the ring, substituents, and the strength of hydrogen-bonding interactions between the phenol and the solvent (*9–12*).

Many essential oils exhibit antioxidant and antimicrobial activities (*13–15*). Phenols, such as thymol, carvacrol, and eugenol, and monocyclic hydrocarbons, such as terpinolene, α-terpinene, and γ-terpinene, belong to the most active natural antioxidants found in the essential oils (*16–18*). However, due to their poor water solubility and the requirement of high concentrations to reach a therapeutic effect, the efficiency of these compounds in treatment is limited.

In this paper, we report on the synthesis of three hydroxymethyl derivatives of thymol, carvacrol, and eugenol as well as a diarylmethane derivate of carvacrol that, to the best of our knowledge, has not been synthesized before. Chemical modifications, such as introducing polar hydroxymethyl moiety into phenol structures, can change antioxidant activity in comparison with the starting compounds (*19*). Hydroxymethylation reactions (nucleophilic addition to the carbonyl group) can be catalyzed by either acids or bases, and electrophilic aromatic substitution reaction of the newly formed hydroxymethylphenol with residual phenol can yield methylenediphenols (*20–22*). The antioxidant properties of prepared derivatives were evaluated, for the first time, by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and Rancimat methods, as well as their antibacterial, antiyeast, and antiproliferative activities and compared to those of the parent compounds.

MATERIALS AND METHODS

General. Synthesized compounds were purified on a silica gel 60 (Kieselgel 60, 0.040–0.063 mm, Merck) column or by recrystallization and analyzed by TLC, mass spectrometry, and NMR analysis. TLC was performed on silica gel 60 precoated plates (Kieselgel 60, thickness = 0.2 mm, Merck). Melting points were obtained using an Kofler Mikroheitztisch apparatus (Reichert) and were uncorrected. Gas chromatograph model 5890 with mass selective detector model 5971A (Hewlett-Packard) was used for purity determination and mass spectral analysis of prepared compounds. High-resolution mass spectra was measured on Waters Micromass Q-ToF micro electrospray ionization spectrometer. NMR experiments were performed on a Bruker AV600 spectrometer equipped with a 5 mm TBI probe with *z*-gradient. Microwell plate reader (Easy-Reader 400 FW, SLT Laboratory Instruments GmbH) was used for antiproliferative activity assay. A Lambda EZ 201 UV–vis spectophotometer from Perkin-Elmer Inc. was used in the DPPH assays. A Rancimat 743 apparatus from Metrohm A.G. was used to measure the induction time of lard with and without additives.

Reagents and Solvents. Thymol (99%), carvacrol (97%), eugenol (99%), and methanal (formaline) were purchased from Fluka Chemie. Butylated hydroxytoluene (BHT), vitamin C, $Na_2HPO_4 \cdot 12H_2O$, and KH_2PO_4 were purchased from Sigma. Solvents were obtained from Merck. Petroleum ether with bp 40–70 °C was used. All solvents were purified prior to use in the experiments.

Gas Chromatography–Mass Spectrometry (GC-MS) Analysis. Analyses were performed on a GC-MS using column HP-101 (dimethylpolysiloxane, Hewlett-Packard): 25 m × 0.2 mm i.d., film thickness = 0.2 μm; column temperature programmed from 70 °C isothermal for 2 min, to 220 °C at a rate of 4 °C min^{-1}; carrier gas, helium; flow rate, 1 mL min^{-1}; injector temperature, 250 °C; volume injected: 1 μL of 1% ether solution of each compound 1:50. MS conditions were as follows: ionization voltage, 70 eV; ion source temperature, 280 °C; mass range, 35–350 mass units.

High-Resolution Mass Spectrometry (HRMS). High-resolution mass spectra were measured in negative ion mode (ESI-): capillary voltage, 3.0 kV; cone voltage, 60 V; collision energy, 6.0 V; desolvation temperature, 200 °C; source temperature, 100 °C; nitrogen flow, 300 L/h. Sample was prepared by dissolving in methanol to the concentration of 10 μg/mL and introduced into the spectrometer with the flow of 5 μL/min. Reference for spectrometer calibration was sodium iodide clusters.

NMR Spectroscopy. The liquid-state one- and two-dimensional ^1H and ^{13}C NMR spectra (600.13 MHz for ^1H, 150.90 MHz for ^{13}C) were measured in DMSO-d_6 at 298 K using standard ^1H, APT, COSY, HSQC, and HMBC techniques. Chemical shifts, in parts per million, were referred to TMS as internal standard. The resolution in ^1H NMR and ^{13}C NMR spectra was 0.29 and 0.54 Hz per point, respectively.

Synthesis of Compounds. *Hydroxymethylation of Carvacrol.* To a solution of 8 g of NaOH (200 mmol) in 100 mL of water and 15 g of carvacrol (100 mmol) was added 10 mL of 35% methanal (ca. 125 mmol) in several portions. Reaction temperature was 50 °C and reaction time, 24 h. The resulting mixture was neutralized with 20% acetic acid (to pH 5) and extracted with ether. The combined ether extracts were concentrated by removal of ether on a rotating evaporator. The residue was submitted to steam distillation to remove residual carvacrol. Water-non-soluble residue was purified by column chromatography on silica gel with petroleum ether/ethyl acetate, Ψ = 3:2 (v/v). Two derivatives of carvacrol, **1** and **2**, were obtained with yields of 15 and 10%.

4-(Hydroxymethyl)-5-isopropyl-2-methylphenol: colorless oil; purity, 96.4% by GC; MS (EI), *m/z* (%) 180 (M^+; 27), 162 (81), 147 (100), 119 (36), 109 (17), 91 (51), 77 (32), 65 (17), 51 (16), 39 (27); ^1H NMR (DMSO-d_6) δ 1.13 (d, 6H, J = 6.7 Hz, H-5″), 2.07 (s, 3H, H-2′), 3.13 ppm (m, 1H, J = 6.7 Hz, H-5′), 4.39 (d, 2H, J = 5.3 Hz, H-4′), 4.77 (t, 1H, J = 5.3 Hz, H-4″), 6.68 (s, 1H, ar-H-6), 6.95 (s, 1H, ar-H-3), 9.00 (s, 1H, H-1′); ^{13}C NMR (DMSO-d_6) δ 15.5 (C-2′), 23.9 (C-5″), 27.6 (C-5′), 60.7 (C-4′), 111.2 (ar-C-6), 120.1 (ar-C-2), 129.0 (ar-C-4), 131.0 (ar-C-3), 144.9 (ar-C-5), 154.5 (ar-C-1).

4,4′-Methylenebis(5-isopropyl-2-methyl)phenol: white crystals, mp 173 ± 0.5 °C; purity, 95.7% by GC; MS (EI), *m/z* (%) 312 (M^+; 13), 269 (2), 162 (100), 161 (18), 147 (12), 121 (6), 91 (5), 77 (3); HRMS (negative ESI), calcd for $C_{21}H_{28}O_2$ [M − H]$^-$ 311.2011, found 311.1996; ^1H NMR (DMSO-d_6) δ 1.09 (d, 12H, J = 7.0 Hz, H-5″), 1.98 (s, 6H, H-2′), 2.96 (m, 2H, J = 7.00 Hz, H-5′), 3.73 (s, 2H, H-4′), 6.50 (s, 2H, ar-H-3), 6.70 (s, 2H, ar-H-6), 8.94 (s, 2H, H-1′); ^{13}C NMR (DMSO-d_6) δ 15.5 (C-2′), 23.6 (C-5″), 28.0 (C-5′), 32.9 (C-4′), 111.4 (ar-C-6), 120.5 (ar-C-2), 127.7 (ar-C-4), 131.4 (ar-C-3), 144.4 (ar-C-5), 153.6 (ar-C-1).

Hydroxymethylation of Eugenol. The same procedure as mentioned above was used. Into a solution of 8 g of NaOH (200 mmol) in 100 mL of water and 15 g of eugenol (100 mmol) was added 10 mL of 35% methanal (ca. 125 mmol) in portions. Reaction temperature was 50 °C and reaction time, 24 h. Thereafter, the resulting mixture was neutralized with 20% acetic acid (until pH 5) and extracted with ether. The combined ether extracts were dried with sodium sulfate, and ether was removed on a rotating evaporator. Derivative was purified by column chromatography on silica gel with petroleum ether/ethyl acetate, Ψ = 3:2 (v/v). The yield was 51%.

4-Allyl-6-(hydroxymethyl)-2-methoxyphenol: colorless oil; purity, 94.5% by GC; MS (EI), *m/z* (%) 194 (M^+; 48), 176 (100), 147 (51), 133 (30), 117 (38), 104 (38), 103 (36), 91 (39), 77 (61), 51 (30); ^1H NMR (DMSO-d_6) δ 3.28 (d, 2H, J = 6.1 Hz, H-4′), 3.77 (s, 3H, H-2′), 4.48 (d, 2H, J = 5.3 Hz, H-6′), 4.98 (t, 1H, J = 5.3 Hz, H-6″), 5.02 (d, 1H, J = 9.9 Hz, H-4‴b), 5.08 (d, 1H, J = 17.0 Hz, H-4‴a), 5.94 (m, 1H, H-4″), 6.66 (s, 1H, ar-H-3), 6.76 (s, 1H, ar-H-5), 8.38 (s, 1H, H-1′); ^{13}C NMR (DMSO-d_6) δ 39.5 (C-4′), 55.8 (C-2′), 58.2 (C-6′), 110.4 (ar-C-3), 115.2 (C-4‴), 119.3 (ar-C-5), 128.8 (ar-C-6), 129.7 (ar-C-4), 138.2 (C-4″), 141.2 (ar-C-1), 146.9 (ar-C-2).

Hydroxymethylation of Thymol. The same procedure was followed as previously described. Ten milliliters of 35% formaldehyde (ca. 125 mmol) in several portions was added to a solution of 8 g of NaOH (200 mmol) in 100 mL of water and 15 g of thymol (100 mmol). Reaction temperature was 50 °C and reaction time, 24 h. The resulting mixture was neutralized with 20% acetic acid (pH 5) and extracted with ether. The combined ether extracts were dried with sodium sulfate, and ether was removed on a rotating evaporator. The residue was purified by two crystallizations from water/ethanol, Ψ = 2:1 (v/v), and from petroleum ether/toluene, Ψ = 1:1 (v/v). The yield was 75%.

4-(Hydroxymethyl)-2-isopropyl-5-methylphenol: white crystals, mp 119.0 ± 0.5 °C; purity, 97.2% by GC; MS (EI), *m/z* (%) 180 (M^+;

53), 165(100), 162 (28), 147 (87), 115 (19), 91 (46), 77 (30), 51 (14); ^{1}H NMR (DMSO-d_6) δ 1.14 (d, 6H, J = 7.0 Hz, H-2″), 2.14 (s, 3H, H-5′), 3.15 (m, 1H, J = 7.0 Hz, H-2′), 4.36 (d, 2H, J = 5.3 Hz, H-4′), 4.76 (t, 1H, J = 5.3 Hz, H-4″), 6.55 (s, 1H, ar-H-6), 7.03 (s, 1H, ar-H-3), 8.96 (s, 1H, H-1′); ^{13}C NMR (DMSO-d_6) δ 17.9 (C-5′), 22.6 (C-2″), 26.0 (C-2′), 61.3 (C-4′), 116.5 (ar-C-6), 125.6 (ar-C-3), 130.4 (ar-C-4), 130.8 (ar-C-2), 133.4 (ar-C-5), 153.0 (ar-C-1).

Free Radical Scavenging Activity (DPPH Assay). The free radical scavenging activity was determined by the DPPH assay (*23*). Fifty microliters of various concentrations of the compounds in ethanol was added to 5 mL of a 0.004% ethanol solution of DPPH. The decrease in absorbance was determined at 517 nm at room temperature at 0 min, 1 min, and every 5 min for 1 h. For each antioxidant concentration tested, the reaction kinetics were plotted and from these graphs the absorbance was read after 30 min. Inhibition of DPPH radical in percentage was calculated in the following way: $I\% = (A_{blank} - A_{sample})/A_{blank} \times 100$, where A_{blank} is the absorbance of the control and A_{sample} is the absorbance of the sample. Sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against sample concentration. Tests were carried out in triplicate.

Induction Period of Lard Oxidation (Rancimat Assay). The lard was prepared by heating a fat (pork meat) at 100 °C, filtered, and stored at −18 °C until further use. The same lard was used for all determinations to obtain good reproducibility. The induction period of lard was determined by Rancimat method (*24*) at 100 °C and an air flow of 20 L/h. Each reaction vial contained 2.5 g of lard, which was filtered prior to the addition of the additives at 60 °C. The additives were dissolved in 2 mL of ethanol as a carrier and added in final concentrations of 0.02 and 0.05% (mass ratio). The reaction vials were vortexed for 20 s before starting the Rancimat measurements. The protection factor (PF) was calculated according to the equation PF = sample induction time/control induction time.

Antimicrobial Activity. Bacteria *Escherichia coli* K-12 (laboratory stock, ordered from *E. coli* Genetic Stock Center) was grown in nutrient broth (NB) medium (8 g of nutrient broth from Difco, 5 g of NaCl, and water added to 1000 mL) and incubated at 37 °C. Solid medium for plates was supplemented with 16 g of agar from BD Bacto. Bacteria were diluted with 66 mM phosphate buffer (per liter: 11.99 g of $Na_2HPO_4 \cdot 12\ H_2O$ and 4.53 g of KH_2PO_4, pH 7.0).

The antimicrobial assay was performed according to the method of Cosentino et al. (*25*) with some modifications. Overnight broth cultures were diluted in 1 mL of fresh NB medium so that the final concentration of cells was approximately 10^6 cfu/mL. Derivatives and parent compounds were then added at various concentrations diluted in ethanol, and suspensions were incubated for 24 h at 37 °C with aeration. Then, bacteria were diluted, and 10 μL spots were plated onto solid NB plates. The plates were incubated at 37 °C for 24 h. Minimal bactericidal concentration (MBC) was defined as the lowest concentration of the compound inhibiting visible growth of *E. coli*. There were two controls included in every test: bacteria in NB media and bacteria with ethanol in NB media. Each experiment was repeated at least two times for each compound at each test concentration.

Antiyeast Activity. The *Saccharomyces cerevisiae* strain used in this study was CEN.PK2 (Mata/Mat. leu2-3,112/leu2-3,112 ura3-52/ura3-52 trp1-289/trp1-289 his3.1/his3.1 MAL2-8c/MAL2-8c SUC2/SUC2; Euroscarf). Cultures were grown with aeration in minimal medium (2% glucose or 2% galactose, 0.17% Difco Yeast Nitrogen Base) supplemented with appropriate amino acids and bases: 40 mg/L adenine, 20 mg/L arginine, 20 mg/L histidine, 100 mg/L leucine, 30 mg/L lysine, 20 mg/L methionine, 100 mg/L threonine, and 20 mg/L tryptophan during the treatment for 2 h at 37 °C in the presence of a range of concentrations of the substances tested. The cells were seeded afterward on the culture plates of the same medium solidified by the addition of 2% agar. After 2 days of incubation at 37 °C, the number of yeast colonies developed on agar were counted. Reduction of colonies after treatment with parent phenols and their derivatives was calculated and expressed as percentage of control colonies that were not exposed to phenols (*26*).

Antiproliferative Activity on Human Carcinoma Cells. The effect of the original compounds and their derivatives on cell mitochondrial activity in vitro was studied on human cervical carcinoma cell line HeLa. HeLa cells were obtained from the American Type Culture Collection. The cells were maintained in RPMI medium (Sigma) with 5% (v/v) fetal calf serum (FCS) (Sigma) in an incubator (Heraeus) at 37 °C, with a humid air atmosphere containing 5% CO_2. The cells were detached from semiconfluent cultures with 0.25% (w/v) trypsin (Sigma) solution for 5 min. Viable cells (upon trypan blue exclusion) were counted on a Bürker-Türk hemocytometer.

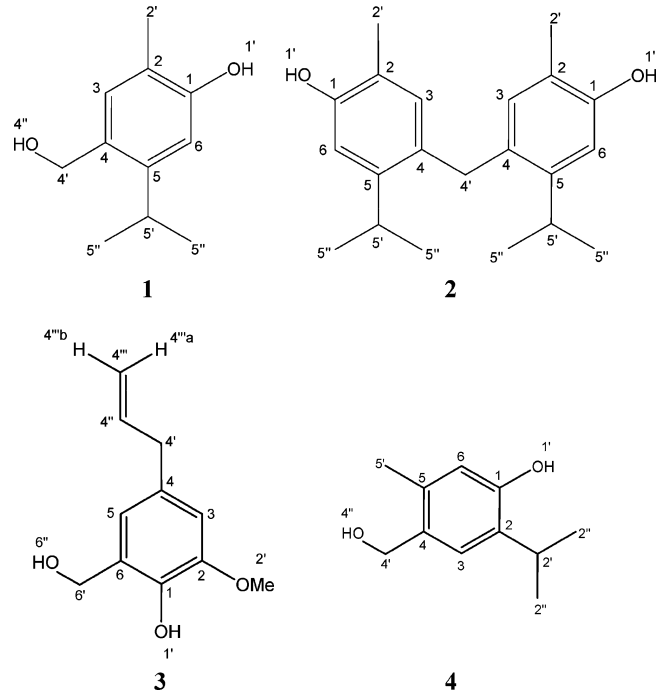


Figure 1. Structures of prepared compounds: 4-(hydroxymethyl)-5-isopropyl-2-methylphenol (**1**); 4,4′-methylenebis(5-isopropyl-2-methyl)phenol (**2**); 4-allyl-6-(hydroxymethyl)-2-methoxyphenol(**3**); 4-(hydroxymethyl)-2-isopropyl-5-methylphenol (**4**).

For the purpose of experiments, the cells were plated at a number of 2×10^4 per well onto 96-microwell plates (200 μL/well) (Greiner) in quadruplicates and incubated for 24 h in RPMI medium with 5% FCS containing different concentrations (0.1–10 mM) of test compounds (*27*). After 24 h, the medium was removed and replaced with 200 μL of Hank's solution without phenol red and 20 μL of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT substrate) solution (EZ4U, Biomedica) (*28*). Microwell plates were incubated at 37 °C for 2 h, and the absorbance was read at 450 nm (measurement wavelength) and 650 nm (reference wavelength) on a microwell plate reader. Statistical analysis was performed using Student's t test. A level of $p < 0.05$ was accepted as statistically significant when compared to control compounds or untreated samples.

RESULTS AND DISCUSSION

Syntheses. Phenols, in alkaline medium, formed phenolates as nucleophiles in reaction with methanal, yielding hydroxymethylphenols. Because charge in the phenolate ion was delocalized between the phenolate oxygen and its respective *ortho*- and *para*- carbons, several products were possible, and three hydroxymethyl phenols were obtained (**Figure 1**): 4-(hydroxymethyl)-5-isopropyl-2-methylphenol (**1**); 4-allyl-2-(hydroxymethyl)-6-methoxyphenol (**3**); and 4-(hydroxymethyl)-2-isopropyl-5-methylphenol (**4**). Furthermore, an electrophilic aromatic substitution reaction of the newly formed hydroxmethylphenol with residual phenol can yield methylenediphenols, and in this way 4,4′-methylenebis(5-isopropyl-2-methylphenol) (**2**) was obtained. All derivatives have no strong odor as their parent compounds. Compounds **3** and **4** were previously synthesized (*22, 29*) according to a similar method, whereas compound **2** was not synthesized before.

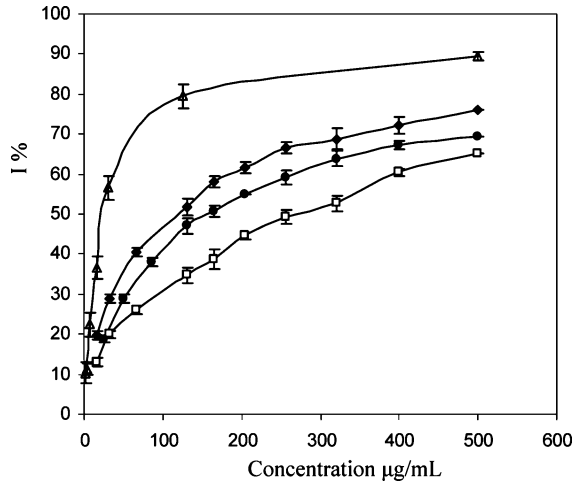


Figure 2. Inhibition ratio (percent) against increasing concentrations of carvacrol (□), carvacrol derivative **1** (●), carvacrol derivative **2** (◆), and BHT (△) in DPPH method. Results were expressed as mean values of triplicates ± SD. IC_{50} (50% inhibition) values were carvacrol, 267 μg/mL; carvacrol derivative **1**, 156 μg/mL; carvacrol derivative **2**, 122 μg/mL; and BHT, 20 μg/mL.

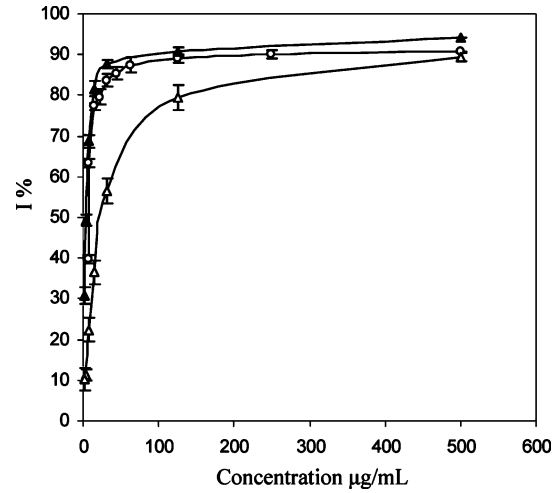


Figure 3. Inhibition ratio (percent) against increasing concentrations of eugenol (○), eugenol derivative **3** (▲), and BHT (△) in DPPH method. Results were expressed as mean values of triplicates ± SD. IC_{50} (50% inhibition) values were eugenol, 9 μg/mL; eugenol derivative **3**, 4 μg/mL; and BHT, 20 μg/mL.

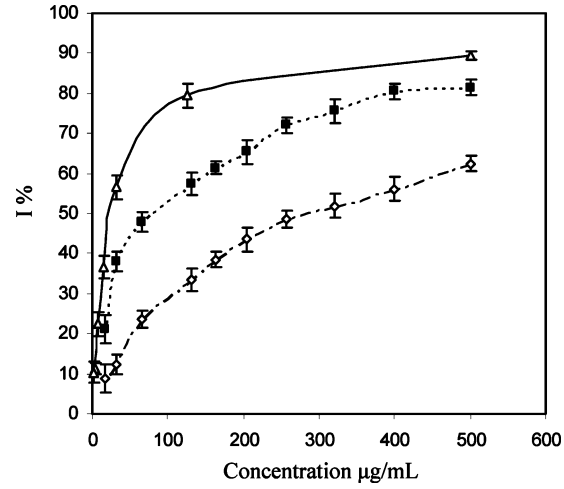


Figure 4. Inhibition ratio (percent) against increasing concentrations of thymol (◇), thymol derivative **4** (■), and BHT (△) in DPPH method. Results were expressed as mean values of triplicates ± SD. IC_{50} (50% inhibition) values were thymol, 269 μg/mL; thymol derivative **4**, 80 μg/mL; and BHT, 20 μg/mL.

Antioxidant Activity. The antioxidant power depends on the chosen method, on the concentration, and on the nature of physicochemical properties of the studied antioxidants (*30*). The rates of formal abstraction of phenolic hydrogen atoms by free radicals are profoundly influenced by the hydrogen-bond-accepting and anion-solvation abilities of solvents, by the electron affinities and the reactivities of radicals, and by the phenol ring substituents (*31*). The resonance stabilization of the unpaired electron is mainly determined by the type and location of the substituents on the aromatic ring.

To evaluate the antioxidation activity of synthesized phenol derivatives, DPPH and Rancimat methods were used. DPPH is a convenient method when radical scavenging abilities of compounds at room temperature are investigated. On the other hand, the Rancimat method was done to evaluate antioxidation potency of compounds under elevated temperature and accelerated aeration in lard. For comparison, starting phenols and well-known antioxidants (BHT and vitamin C) were also investigated by the same methods.

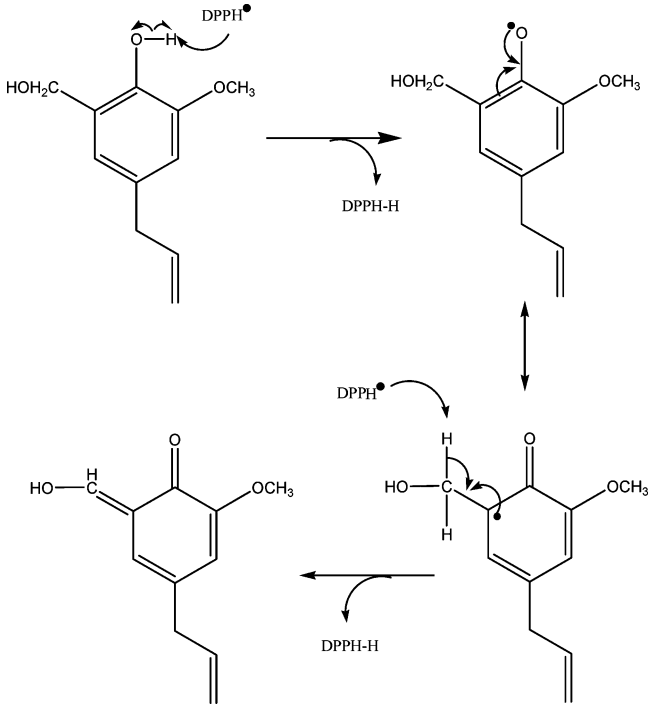
Antioxidant Activity According to the DPPH Radical Scavenging Method. It is accepted that the scavenging mechanism of reaction is abstracting a hydrogen atom from a phenol donor to give DPPH-H and a phenoxy radical. Ingold et al. (*31, 32*) measured the kinetics of free radical reactions and confirmed that there are large solvent effects on the rates of hydrogen abstraction from O−H bonds: deactivation of the substrate when it can act as a hydrogen-bond-donor to a hydrogen-bond-accepting solvent (kinetic solvent effect) that is independent of DPPH.

Thymol, carvacrol, and eugenol derivatives scavenged the DPPH radical in a dose-dependent manner, and the DPPH radical scavenging activity (IC_{50}) was decreased in the following order: eugenol derivative **3** > eugenol > BHT > thymol derivative **4** > carvacrol derivative **2** > carvacrol derivative **1** > carvacrol > thymol. Results are presented in **Figures 2**–**4**.

Thymol with greater steric hindrance of the phenolic group in comparison to carvacrol had higher antioxidant activity, as was found in lipids (*16*). It is known that compounds with a hydroxyl group sterically hindered, such as BHT, possess a high antioxidative activity (*33*). Eugenol and BHT reduce two or more DPPH radicals, despite the availability of only one hydrogen on a hydroxyl group, and there are three suggested hypotheses to explain the antiradical efficiencies of the different monophenolic compounds (*23*).

When synthesized phenol derivatives were investigated, they all showed better antioxidant properties in comparison with starting compounds. Namely, the substitution radical of 1,2- or 1,4-orientation generally donates an electron to the aromatic ring to activate it, either by the resonance effect or by inductive effect, but substitution of the radical in the 1,3-orientation tends to inactivate the ring (*34*). Therefore, the introduction of a hydroxymethyl substituent in 1,2-orientation in derivative **3** and in 1,4-orientation in derivatives **1** and **4** was important for antioxidant activity. Thymol derivative **4**, probably due to the steric effect of isopropyl group, exhibited better antioxidant activity in comparison with carvacrol derivative **1**. Derivative **3** was found to be most effective free radical scavenger, even better than BHT and eugenol. The contribution of the hydroxymethyl group in additional hydrogen transfer to DPPH radical in derivatives **1**, **3**, and **4** in comparison to the parent compounds can be suggested as shown in **Scheme 1**. The

Scheme 1. Potential Contribution of the Hydoxymethyl Group to DPPH Radical Scavenging Activity



inhibitory effect of dimerized compound of carvacrol **2** was higher than that of carvacrol and its derivative **1** with a hydroxymethyl moiety. Derivative **2** can form a stable radical with diphenylmethane structure that could contribute to its antioxidative properties.

Antioxidant Activity According to the Rancimat Method. The mechanism of this method is reported to be based on measuring the changes of electrical conductivity of water caused by the formation of short-chain compounds when fats and oils are oxidized under elevated temperature (100 °C) and accelerated aeration (an air flow of 20 L/h). The higher the induction times of lard compared to the control, the better is the antioxidant activity of that compound. All synthesized derivatives, as well as starting phenols, BHT and vitamin C, were tested at final concentrations of 0.02 and 0.05% (mass ratio). Results are presented in **Table 1**.

All tested compounds, as in the previously discussed DPPH method, resulted in an increase of protection factor or induction period. All derivatives are remarkably better antioxidants in comparison to the corresponding starting compounds. At the elevated temperature of the Rancimat method it is reasonable to expect that parent compounds were lost quickly, whereas the derivatives with larger molecular weight and added hydroxymethyl group (or diarylmethane derivative) were retained longer in the reaction medium. Eugenol derivative **3** is the best antioxidant among all derivatives according to the Rancimat method, and at the concentration of 0.05% in lard this compound is better than BHT or vitamin C. The derivative of thymol (**4**) is also a potent antioxidant. Antioxidant activity for the concentration of all tested compounds in lard of 0.02% decreased in the order BHT > thymol derivative **4** > eugenol derivative **3** > carvacrol derivative **2** > vitamin C > carvacrol derivative **1** > eugenol > carvacrol > thymol. The order of antioxidant activity for the concentration of compounds in lard of 0.05% is slightly different: eugenol derivative **3** > BHT > thymol derivative **4** > carvacrol derivative **2** > carvacrol derivative **1**, vitamin C > eugenol > thymol > carvacrol.

Table 1. Protection Factors (PF) of Parent Phenols and Synthesized Derivatives Depending on Their Concentration in Lard[a]

sample	concentration (%)	PF
control[b]	0	1.00
carvacrol	0.02	1.37
	0.05	1.48
carvacrol derivative **1**	0.02	1.55
	0.05	2.54
carvacrol derivative **2**	0.02	2.10
	0.05	2.82
eugenol	0.02	1.39
	0.05	1.67
eugenol derivative **3**	0.02	2.45
	0.05	5.84
thymol	0.02	1.32
	0.05	1.59
thymol derivative **4**	0.02	2.61
	0.05	4.15
BHT	0.02	3.55
	0.05	4.63
vitamin C	0.02	1.91
	0.05	2.54

[a] The results are means of two different experiments. [b] Control = pure lard without additives.

Antibacterial Activity against Wild Type *E. coli* K-12. The addition of low, medium, or high concentrations of parent phenols and their derivatives into bacterial suspensions had no effect on the viability of *E. coli* after 40 min of incubation (data not shown), which is different from other reported work in which a quick effect on bacterial survival was observed for whole essential oils (*35*). In the time course experiment with fixed concentrations of added phenols and their derivatives of 0.75 and 2 mM, respectively, the maximal toxic effect of derivatives was observed after 4 h of incubation. Further incubation of treated cells to 24 h caused the recovery of cell survival to almost 100%, except for carvacrol (data not shown). After 24 h of incubation, the antimicrobial effect was observed with most investigated compounds at different final concentrations (**Table 2**). Carvacrol derivatives **1** and **2** at a final concentration of 10 mM had no toxic effect on the viability of *E. coli*, whereas other derivatives showed reduced antibacterial effect compared to the original compounds. At the same compound concentrations, *E. coli* cells were more sensitive compared to yeast cells (**Table 3**).

Antiyeast Activity. All derivatives showed lower cytotoxic effect for (*S. cerevisiae*) yeast cells compared to the parent compounds (**Table 3**). The 50% lethal dose (LD_{50}) determined by reduction of yeast colonies showed also the lower cytotoxic effect for derivatives compared to the parent compounds. Carvacrol derivative **2** showed the lowest cytotoxic effect compared to other parent phenols and their derivatives, but that cytotoxic effect was the same as the cytotoxic effect of BHT (**Table 4**).

Antiproliferative Activity for Tumor Cells. The effect of carvacrol and carvacrol derivatives **1** and **2** on mitochondrial activity of HeLa cells is shown in **Figure 5**. All substances caused a dose-dependent decrease in mitochondrial activity of HeLa cells when compared to the control, untreated cells

Table 2. Antimicrobial Activity of Phenols and Their Derivatives against Wild Type *Escherichia coli* K-12[a]

compound	concentration (mM)	reduction of colonies (%)	LD_{50} (mM)	MBC 24 h (mM)
carvacrol	0.5	25[b]	between 0.5 and 1	2
	1	76		
carvacrol derivative **1**	5	52	5	>10
	10	73		
carvacrol derivative **2**	5	0	>10	>10
	10	0		
eugenol	1	46	between 1 and 2	5
	2	73		
eugenol derivative **3**	2	33	between 2 and 5	7
	5	86		
thymol	0.5	50	0.5	2
	1	73		
thymol derivative **4**	2	10	between 2 and 5	7
	5	75		
EtOH	2	5	>7	>7
	7	10		

[a] The results are means of two different experiments. [b] Number of colonies in control sample without phenols is 21.

Table 3. Yeast (*Saccharomyces cerevisiae*) Growth after Treatment with Phenols and Their Derivatives for 2 h[a]

	concentration					
compound	1 mM	2 mM	5 mM	10 mM	20 mM	40 mM
carvacrol	++[b]	0	0	0	0	0
carvacrol derivative **1**	+++	+++	+++	+	0	0
carvacrol derivative **2**	+++	+++	+++	++	+	0
eugenol	+++	++	+	0	0	0
eugenol derivative **3**	+++	+++	+++	+	0	0
thymol	++	0	0	0	0	0
thymol derivative **4**	+++	+++	++	0	0	0

[a] The results are the means of three different experiments. [b] +++, no difference compared to control medium; ++, gently reduced growth; +, high reduction in growth; 0, no live cells.

(carvacrol, 0.5–10 mM, $p < 0.05$; carvacrol derivative **1**, 5–10 mM, $p < 0.05$; carvacrol derivative **2**, 5–10 mM, $p < 0.05$). Derivative **1** stimulated mitochondrial activity in lower concentrations (0.5–1 mM, $p < 0.05$). If effect on mitochondrial activity was compared to that of the original compound, carvacrol, the effect of derivative **2** on mitochondrial activity was lower (0.5–10 mM, $p < 0.05$), whereas **1** had a stimulating activity in low concentration range (0.5 mM, $p < 0.05$). The effects in the concentration range of 1–10 mM were the same as those of carvacrol. Hence, carvacrol derivative **2** showed lower inhibiting bioactivity for the HeLa cells as was also noted for the particular derivative when used on yeast and bacteria (**Tables 2** and **3**).

The effect of eugenol and eugenol derivative **3** on mitochondrial activity of HeLa cells is shown in **Figure 6**. Both substances caused a dose-dependent decrease in mitochondrial activity of HeLa cells with the same efficiency (0.5–10 mM, $p < 0.05$) when compared to the control, untreated cells. If the effect of the derivative was compared to that of the original compound, eugenol, 1 mM eugenol derivative had a slightly lower effect on mitochondrial activity ($p > > 0.05$).

Table 4. Number of Yeast Colonies after Treatment with Phenols and Their Derivatives and Approximate LD_{50}[a]

substance	concentration (mM)	no. of colonies	reduction of colonies (%)	LD_{50} (mM)
none		39	0	
carvacrol	1	21	46	1
carvacrol derivative **1**	5	37	5	
carvacrol derivative **1**	10	12	69	between 5 and 10
carvacrol derivative **2**	10	27	31	
carvacrol derivative **2**	15	0	100	between 10 and 15
eugenol	2	31	30	
eugenol	5	0	100	between 2 and 5
eugenol derivative **3**	5	35	10	
eugenol derivative **3**	10	5	87	between 5 and 10
thymol	1	20	49	1
thymol derivative **4**	5	23	40	>5
BHT	1	36	8	
BHT	5	27	31	
BHT	10	24	38	>10
EtOH	5%	41	0	
EtOH	15%	32	18	>15%

[a] Results are means of three different experiments.

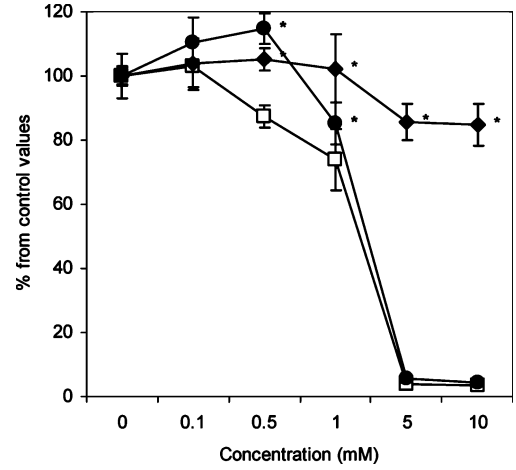


Figure 5. Mitochondrial activity (percent) against increasing concentrations of carvacrol (□), carvacrol derivative **1** (●), and carvacrol derivative **2** (◆) in MTT assay. Results were expressed as mean values of quadruplicates ± SD. Significance was calculated according to the Student's *t* test, compared to the control compound, carvacrol; ∗, $p < 0.05$.

The effect of thymol and thymol derivative **4** on mitochondrial activity of HeLa cells is shown in **Figure 7**. Both substances caused dose-dependent decreases in mitochondrial activity of HeLa cells when compared to the control, untreated cells (for thymol, 0.1–10 mM, $p < 0.05$; for thymol derivative **4**, 0.5–10 mM, $p < 0.05$). If the effect of derivative was compared to that of the parent compound, thymol, there was no difference (for all concentrations, $p > 0.05$).

Summarizing results obtained for the HeLa cells treated by the new derivatives in comparison to the parent substances, we conclude that only carvacrol derivative **2** showed lower inhibiting capacity, which makes this particular derivative attractive as an efficient antioxidant with negligible cytotoxic effects.

However, the finding of retained inhibiting effects for all other derivatives in comparison to the parent substances for human malignant HeLa cells (uterine cervical carcinoma) makes these substances also potentially attractive as antioxidants with anticancer capacities, which will be further studied. Namely, although cytotoxicity of oxygen free radicals is essential for the therapeutic effects of various cancer therapies (such as radiotherapy or doxorubicin chemotherapy), malignant cells

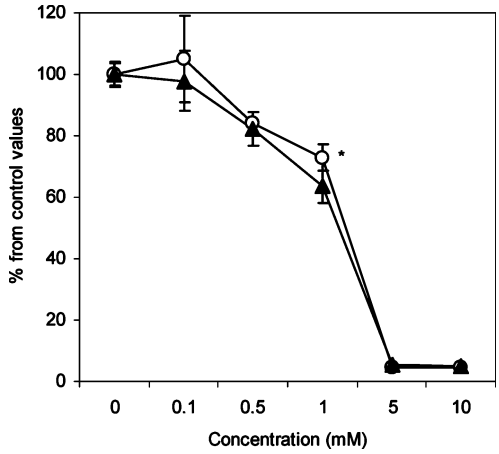


Figure 6. Mitochondrial activity (percent) against increasing concentrations of eugenol (○) and eugenol derivative **3** (▲) in MTT assay. Results were expressed as mean values of quadruplicates ± SD. Significance was calculated according to Student's *t* test, compared to the control compound, eugenol; ∗, $p < 0.05$.

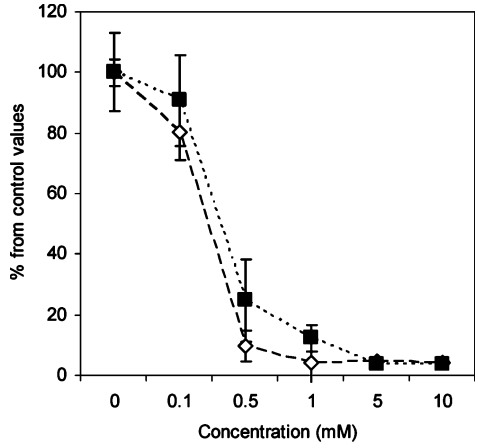


Figure 7. Mitochondrial activity (percent) against increasing concentrations of thymol (◇) and thymol derivative **4** (□) in MTT assay. Results were expressed as mean values of quadruplicates ± SD. Significance was calculated according to Student's *t* test, compared to the control compound, thymol; ∗, $p < 0.05$.

often avoid cytotoxicity of the thus induced oxidative stress, which, on the other hand, shows undesirable cytotoxicity for nonmalignant cells. Therefore, antioxidants that are toxic for cancer cells but do not affect other types of cells (as observed for bacteria and yeast in this study) would be attractive not only to study mechanisms of the cancer cell defense against toxicity of free radicals but also to be used in potential adjuvant cancer therapies (*36*). Because it is assumed that cancer cells show relative resistance to cytotoxicity of oxidative stress because of the less expressed lipid peroxidation, we aim to study further biological activities of these derivatives on other malignant and nonmalignant cell types, in particular using rat stem-cell-like liver cells and the yeast mutant producing polyunsaturated fatty acids, which could give further information about the bioactivity principles of the novel carvacrol, thymol, and eugenol derivatives (*37, 38*). Thus, we aim to analyze if these derivatives express higher toxicity for malignant than for nonmalignant cells, which would make them attractive for experimental adjuvant cancer treatments.

Conclusion. A series of modified phenols were obtained by simple chemical synthesis, of which the diarylmethane derivative of carvacrol to the best of our knowledge was prepared for the first time. All derivatives are better antioxidants than the corresponding parent compounds according to DPPH and Rancimat measurements. Eugenol derivative **3** is a remarkably better antioxidant in comparison to other derivatives. Derivatives have lower antiproliferative activity and toxicity than starting compounds, in particular, carvacrol derivative **2**, which makes this particular derivative attractive as an efficient antioxidant with negligible cytotoxic effects. Increased antioxidant capacities and reduced cytotoxicy make the synthesized derivatives attractive for possible use as antioxidants that might be tested for potential use in prevention and/or adjuvant therapies of disorders associated with oxidative stress.

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