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Synthesis, Antioxidant and Antimicrobial Evaluation of Simple Aromatic Esters of Ferulic Acid

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Aromatic ester derivatives of ferulic acid where the phenolic hydroxyl is free (**6a-d**) or acetylated (**5a-d**) were evaluated for their antioxidant and antimicrobial properties. The superoxide radical scavenging capacity of compounds **5d** and **6d-e** (IC_{50} of 0.19, 0.27 and 0.20 mM, respectively) was found to be twice as active as α-tocopherol (IC_{50} = 0.51 mM). DPPH radical scavenging capacity was moderate and only found in compounds bearing free phenolic hydroxyl groups (**6a-e**). With regard to antimicrobial properties, compounds **6b** and **6c** displayed significant activity against *Enterococcus faecalis* (MICs = 16 μg/mL) and vancomycin-resistant *E. faecalis* (MIC for **6b**, 32 and for **6c**, 16 μg/mL). Compound **6c** also demonstrated prominent activity against planktonic *Staphylococcus aureus* with a MIC value of <8 μg/mL and it inhibited bacterial biofilm formation by *S. aureus* with a MBEC value of <8 μg/mL, which was 64 and 128 times more potent than ofloxacin and vancomycin, respectively.

Key words: Ferulic acid, DPPH, Superoxide, Antimicrobial, Biofilm

INTRODUCTION

Ferulic acid (FA) (**3**), 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid, acts as an effective radical scavenger due to its ability to form a resonance stabilized phenoxy radical with its phenolic nucleus and its extended side-chain conjugation (Graf, 1992). The various ester forms of FA are known to behave as potent antioxidants in plants and plant-derived foods by trapping and stabilizing radical species (Masuda et al., 2006). Since researchers has been able to produce FA in mass quantities from rice bran, the utilization of this dietary phenolic compound for synthetic approaches has become very attractive to develop FA-derived novel chemopreventive strategies as antioxidant and anticancer agents (Mori et al., 1999; Taniguchi et al., 1999). For example, different alkyl esters of FA have recently emerged as synthetic agents with antioxidant and radical scavenging activities bearing potential for treatment of inflammation and cancer (Murakami et al., 2002; Nenadis et al., 2003; Jayaprakasam et al., 2006). These types of lipophilic FA derivatives have also been shown to inhibit phorbol ester-induced Epstein-Barr virus activation and superoxide anion generation in cellular systems (Murakami et al., 2000). Moreover, FA is reported to have antimicrobial activity against Gram-positive, Gram-negative bacteria and yeasts (Jeong et al., 2000) in which the antimicrobial mechanism of FA was attributed to its inhibition of arylamine *N*-acetyltransferase in the bacteria (Lo and Chung, 1999). In support of these findings, the antimicrobial activity of the synthetic alkyl ferulates (Michiyo et al., 2002; Ou and Kwok, 2004) and some phenolic plant extracts containing FA have also been reported (Panizzi et al., 2002; Proestos et al., 2005). In addition, geranylation of the phenolic group in FA was shown to inhibit bacterial biofilm formation by *Porphyromonas gingivalis* and *Streptococcus* mutants (Bodet et al., 2008). Considering the promising results with alkyl ferulates, we now prepared simple aromatic ester derivatives of FA (Table I) and assessed their antioxidant potential and antimicrobial activities.

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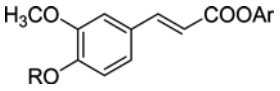
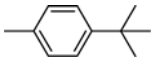
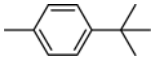
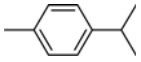
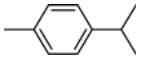
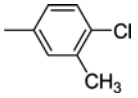
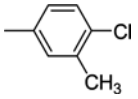
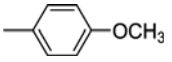
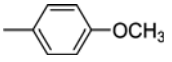
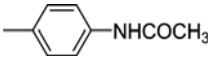
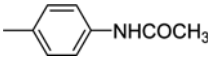
Table I. Synthesized aromatic esters of ferulic acid



Compound	R	Ar
3 (FA)	H	H
4	Ac	H
5a	Ac	
6a	H	
5b	Ac	
6b	H	
5c	Ac	
6c	H	
5d	Ac	
6d	H	
5e	Ac	
6e	H	

MATERIALS AND METHODS

Vanillin, malonic acid, hydrazine hydrate, dimethylaminopyridine (DMAP), triethylamine (TEA), oxalyl chloride, aniline, acetic anhydride, pyridine and phenol derivatives were purchased from Aldrich Chemical and Merck Chemical. Xanthine, xanthine oxidase, cytochrome c, 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene, α-tocopherol, thiobarbituric acid (TBA) were purchased from Sigma Chemical. IR (KBr) spectra were recorded on a Mattson 1000 FTIR spectrometer. ^{1}H-NMR spectra were recorded in DMSO-d_6 on a Varian Mercury 400, 400 MHz High Performance Digital FT-NMR spectrometer using tetramethylsilane as the internal standard at the NMR facility of the Faculty of Pharmacy, Ankara University. All chemical shifts were recorded as δ (ppm). High resolution mass spectra (HRMS) were performed on a Waters LCT Premier XE orthogonal acceleration time-of-flight (oa-TOF) mass spectrometer with using ESI (+) or ESI (–) methods (Waters Corporation). Melting points were determined with an SMP-II Digital Melting Point Apparatus and are uncorrected. Flash chromatography was performed with a Combiflash®Rf automated flash chromatography system (Teledyne-Isco) using hexane-ethyl acetate or dichloromethane-methanol solvent gradients. Final purity of the compounds before biological testing was determined by ultra pressure liquid chromatography (UPLC) with UV detection using acetonitrile/water solvent gradient.

Synthesis of FA (3)

FA was synthesized by Knoevenagel condensation between vanillin and malonic acid as previously reported (Xia et al., 2008). M.p. 171-172ºC; lit. 172-173ºC (Xia et al., 2008). IR (KBr, cm^{-1}): 3440 (O-H), 1696 (C=O). HRMS *m/z* $C_{10}H_{10}O_4$ (M-1): calcd. 193.0501; found: 193.0503.

Synthesis of O-acetyl FA (4)

O-acetyl FA was prepared by the reaction of FA with acetic anhydride in pyridine using DMAP as a catalyst. M.p. 199-202ºC; lit. 199-200ºC (Galey and Terranova, 1996). IR (KBr, cm^{-1}): 3274-2037 (broad, COOH), 1770 (C=O), 1690 (C=O). HRMS $C_{12}H_{12}O_5$ (M+1): calcd. 237.0763; found: 237.0764.

General synthesis of esters of O-acetyl FA (5a-e)

To a mixture of *O*-acetyl FA (1 mmol) in dry dichloromethane (DCM; 5 mL), oxalyl chloride (2 mmol) and DMF (25 μL) were added, and the resulting solution was stirred under argon atmosphere for 15 min at rt. After evaporation of the solvent, the residue was dissolved in dry DCM (5 mL) and the appropriate phenol derivative (1 mmol) and TEA (2.5 mmol) were added. The reaction mixture was stirred for 45 min at rt under argon atmosphere. After addition of ice-water, the pH of the medium was adjusted to 4-5 with acetic acid. The separated organic phase was washed with water, dried over anhydrous Na_2SO_4 and the solvent was evaporated under vacuum. The residue was purified by automated flash column chromatography using hexane-EtOAc or DCM-MeOH as eluents.

4-Tert-butylphenyl 3-(4-acetyloxy-3-methoxyphenyl)-2-propenoate (5a)

Elution with hexane-EtOAc 0-25% afforded **5a** as a white solid (yield 74%); m.p. 156-157ºC; IR (KBr, cm^{-1}): 1776 (C=O), 1728 (C=O); ^{1}H-NMR (DMSO-d_6): δ 1.30 (s, 9H, $(CH_3)_3$), 2,27 (s, 3H, $COCH_3$), 3.84 (s, 3H, OCH_3), 6.96 (d, *J* = 16 Hz, 1H, α-H), 7.13 (d, *J* = 8.8 Hz, 2H, ArH), 7.17 (d, *J* = 8.4 Hz, 1H, ArH), 7.39 (dd, *J* = 8.4, 1.6 Hz, 1H, ArH), 7.45 (d, *J* = 8.8 Hz, 2H, ArH), 7.62 (d, *J* = 1.6 Hz, 1H, ArH), 7.84 (d, *J* = 16 Hz, 1H, β-H); HRMS for $C_{22}H_{24}O_5$ (M+1): calcd. 369.1702; found: 369.1709.

4-Iso-propylphenyl 3-(4-acetyloxy-3-methoxyphenyl)-2-propenoate (5b)
Elution with hexane-EtOAc 0-25% afforded **5b** as a white solid (yield 77%); m.p. 132-133ºC; IR (KBr, cm^{-1}): 1770 (C=O), 1728 (C=O); ^{1}H-NMR (DMSO-d_6): δ 1.19 (d, *J* = 6.8 Hz, 6H, $(CH_3)_2$), 2.25 (s, 3H, $COCH_3$), 2.90 (m, 1H, C*H*$(CH_3)_2$), 3.82 (s, 3H, OCH_3), 6.93 (d, *J* = 16 Hz, 1H, α-H), 7.09 (m, 2H, ArH), 7.14 (d, *J* = 8.0 Hz, 1H, ArH), 7.28 (m, 2H, ArH), 7.36 (dd, *J* = 8.4, 2.0 Hz, 1H, ArH), 7.60 (d, *J* = 2 Hz, 1H, ArH), 7.81 (d, *J* = 16 Hz, 1H, β-H); HRMS for $C_{21}H_{22}O_5$ (M+1): calcd. 355.1545; found: 355.1535.

(4-chloro-3-methyl)Phenyl 3-(4-acetyloxy-3-methoxyphenyl)-2-propenoate (5c)
Elution with hexane-EtOAc 0-25% afforded **5c** as white solid (yield 70%); m.p. 122-123ºC; IR (KBr, cm^{-1}): 1765 (C=O), 1728 (C=O); ^{1}H-NMR (DMSO-d_6): δ 2.25 (s, 3H, $COCH_3$), 2.32 (s, 3H, CH_3), 3.82 (s, 3H, OCH_3), 6.93 (d, *J* = 16 Hz, 1H, α-H), 7.08 (dd, *J* = 8.8, 2.8 Hz, 1H, ArH), 7.15 (d, *J* = 8.0 Hz, 1H, ArH), 7.24 (d, *J* = 2.8 Hz, 1H, ArH), 7.37 (dd, *J* = 8.4, 1.6 Hz, 1H, ArH), 7.46 (d, *J* = 8.8 Hz, 1H, ArH), 7.60 (d, *J* = 1.6 Hz, 1H, ArH), 7.83 (d, *J* = 15.6 Hz, 1H, β-H); HRMS for $C_{19}H_{17}ClO_5$ (M+1): calcd. 361.0843; found: 361.0836.

4-Methoxyphenyl 3-(4-acetyloxy-3-methoxyphenyl)-2-propenoate (5d)
Elution with DCM-MeOH 0-2% afforded **5d** as a white solid (yield 60%); m.p. 151-152ºC; IR (KBr, cm^{-1}): 1770 (C=O), 1744 (C=O); ^{1}H-NMR (DMSO-d_6): δ 2.27 (s, 3H, $COCH_3$), 3.77 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 6.93 (d, *J* = 16 Hz, 1H, α-H), 6.98 (m, 2H, ArH), 7.13 (m, 2H, ArH), 7.17 (d, *J* = 8.4 Hz, 1H, ArH), 7.38 (dd, *J* = 8.4, 2 Hz, 1H, ArH), 7.62 (d, *J* = 1.6 Hz, 1H, ArH), 7.83 (d, *J* = 16 Hz, 1H, β-H); HRMS for $C_{19}H_{18}O_6$ (M+1): calcd. 343.1182; found: 343.1181.

4-(acetylamino)Phenyl 3-(4-acetyloxy-3-methoxyphenyl)-2-propenoate (5e)
Elution with DCM-MeOH 0-5% afforded **5e** as beige solid (yield 70%); m.p. 234-235ºC; lit. 246-250ºC (Del Soldato et al., 2002). IR (KBr, cm^{-1}): 3376 (N-H), 1744 (C=O), 1722 (C=O), 1685 (C=O); ^{1}H-NMR (DMSO-d_6): δ 2.05 (s, 3H, $COCH_3$), 2.27 (s, 3H, $COCH_3$), 3.84 (s, 3H, OCH_3), 6.94 (d, *J* = 16 Hz, 1H, α-H), 7.14 (m, 2H, ArH), 7.17 (d, *J* = 8.0 Hz, 1H, ArH), 7.38 (dd, *J* = 8.4, 1.6 Hz, 1H, ArH), 7.61-7.62 (m, 3H, ArH), 7.84 (d, *J* = 16 Hz, 1H, β-H); HRMS for $C_{20}H_{19}O_6$ (M+1): calcd. 370.1291; found: 370.1277.

Synthesis of esters of FA (6a-e)

To a solution of the appropriate *O*-acetyl FA ester derivative (**5a-e**) (0.5 mmol) in acetonitrile (5 mL) was added hydrazine monohydrate (1.5 mmol), and stirred for 15 min at RT. After addition of acetic acid and water to the reaction mixture, the precipitate formed was purified by recrystallization from the appropriate solvent.

4-Tert-butylphenyl 3-(4-hydroxy-3-methoxyphenyl)-2-propenoate (6a)
Crystallization from MeOH-water afforded **6a** as a white solid (yield 83%); m.p. 160-161ºC; IR (KBr, cm^{-1}): 3557 (O-H), 1722 (C=O); ^{1}H-NMR (DMSO-d_6): δ 1.30 (s, 9H, $(CH_3)_3$), 3.83 (s, 3H, OCH_3), 6.72 (d, *J* = 16 Hz, 1H, α-H), 6.82 (d, *J* = 8.0 Hz, 1H, ArH), 7.09 (d, *J* = 8.8 Hz, 2H, ArH), 7.20 (dd, *J* = 8.4, 1.6 Hz, 1H, ArH), 7.43 (m, 3H, ArH), 7.74 (d, *J* = 16 Hz, 1H, β-H); HRMS for $C_{20}H_{22}O_4$ (M+1): calcd. 327.1596; found: 327.1586.

4-Iso-propylphenyl 3-(4-hydroxy-3-methoxyphenyl)-2-propenoate (6b)
Crystallization from methanol-water mixture afforded **6b** as a white solid (yield 55%); m.p. 134-135ºC; IR (KBr, cm^{-1}): 3509 (O-H), 1722 (C=O); ^{1}H-NMR (DMSO-d_6): δ 1.21 (d, *J* = 6.4 Hz, 6H, $(CH_3)_2$), 2.92 (m, 1H, C*H*$(CH_3)_2$), 3.83 (s, 3H, OCH_3), 6.71 (d, *J* = 15.6 Hz, 1H, α-H), 6.82 (d, *J* = 7.6 Hz, 1H, ArH), 7.09 (d, *J* = 8.8 Hz, 2H, ArH), 7.20 (dd, *J* = 8.4, 1.6 Hz, 1H, ArH), 7.29 (d, *J* = 8.8 Hz, 2H, ArH), 7.42 (d, *J* = 1.6 Hz, 1H, ArH), 7.73 (d, *J* = 15.6 Hz, 1H, β-H), 9.70 (bs, 1H, OH); HRMS for $C_{19}H_{20}O_4$ (M+1): calcd. 313.1440; found: 313.1434.

(4-chloro-3-methyl)Phenyl 3-(4-hydroxy-3-methoxyphenyl)-2-propenoate (6c)
Crystallization from MeOH-water afforded **6c** as a beige solid (yield 60%); m.p. 113-114ºC; IR (KBr, cm^{-1}): 3434 (O-H), 1717 (C=O); ^{1}H-NMR (DMSO-d_6): δ 2.34 (s, 3H, CH_3), 3.83 (s, 3H, OCH_3), 6.71 (d, *J* = 16 Hz, 1H, α-H), 6.82 (d, *J* = 8.0 Hz, 1H, ArH), 7.07 (dd, *J* = 8.4, 2.8 Hz, 1H, ArH), 7.20-7.23 (m, 2H, ArH), 7.42 (d, *J* = 2.0 Hz, 1H, ArH), 7.47 (d, *J* = 8.8 Hz, 1H, ArH), 7.75 (d, *J* = 16 Hz, 1H, β-H), 9.75 (bs, 1H, OH); HRMS for $C_{17}H_{15}ClO_4$ (M+1): calcd. 319.0737; found: 319.0739.

4-Methoxyphenyl 3-(4-hydroxy-3-methoxyphenyl)-2-propenoate (6d)
6d was obtained as a white solid (yield 88%); m.p. 146-147ºC; IR (KBr, cm^{-1}): 3418 (O-H), 1733 (C=O); ^{1}H-NMR (DMSO-d_6): δ 3.76 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 6.69 (d, *J* = 15.6 Hz, 1H, α-H), 6.82 (d, *J* = 8.0 Hz, 1H, ArH), 6.97 (m, 2H, ArH), 7.10 (m, 2H, ArH), 7.20 (dd, *J* = 8.0, 2.0 Hz, 1H, ArH), 7.41 (d, *J* = 2.0 Hz, 1H, ArH), 7.73 (d, *J* = 16 Hz, 1H, β-H); HRMS for

$C_{17}H_{16}O_5$ (M+1): calcd. 301.1076; found: 301.1078.

4-(acetylamino)Phenyl 3-(4-hydroxy-3-methoxyphenyl)-2-propenoate (6e)

Crystallization from MeOH-water afforded **6e** as beige solid (yield 72%); m.p. 194-197°C; lit. 185-195°C (Del Soldato et al., 2002). IR (KBr, cm^{-1}): 3445-3200 (N-H, O-H), 1733 (C=O), 1674 (C=O); ^{1}H-NMR (DMSO-d_6): δ 2.02 (s, 3H, $COCH_3$), 3.81 (s, 3H, OCH_3), 6.67 (d, J = 15.6 Hz, 1H, α-H), 6.79 (d, J = 8.4 Hz, 1H, ArH), 7.08 (d, J = 9.2 Hz, 2H, ArH), 7.18 (dd, J = 8.0, 2.0 Hz, 1H, ArH), 7.39 (d, J = 1.6 Hz, 1H, ArH), 7.58 (d, J = 9.2 Hz, 2H, ArH), 7.71 (d, J = 15.6 Hz, 1H, β-H), 9.68 (bs, 1H, OH), 9.98 (bs, 1H, NH); HRMS for $C_{18}H_{17}NO_5$ (M+1): calcd. 328.1185; found: 328.1189.

Antioxidant activity

Superoxide anion radical ($O_2^{\bullet-}$) scavenging activity

The capacity of FA derivatives to scavenge $O_2^{\bullet-}$ was determined spectrophotometrically on the basis of inhibition of cytochrome c reduction according to the modified method of McCord and Fridovich (McCord and Fridovich, 1969). $O_2^{\bullet-}$ was generated by a xanthine-xanthine oxidase system. The incubation mixture (1 mL, total volume) consisted of phosphate buffer (pH 7.8, 0.05 M), xanthine oxidase (0.32 Units/mL), xanthine (50 μM), cytochrome c (60 mM) and different concentrations of FA derivatives at 100 μL. The reaction was initiated by the addition of xanthine oxidase to this mixture. The absorbance was measured at 550 nm for 3 min (cytochrome c reduction). Each experiment was performed in triplicate and the results are expressed as a percent of the control (DMSO).

DPPH radical scavenging assay

DPPH assays were performed using purified samples of test compounds as previously described (Blois, 1958). Test samples were dissolved in DMSO and mixed with methanol solutions of DPPH (100 mM) in 96-well micro titer plates, followed by incubation at 37°C for 30 min. DPPH reduction was estimated at 517 nm. For each test compound, different concentrations were tested. Final concentrations of test materials were typically in the range from 0.0675 to 1 mM. Percent inhibition by sample treatment was determined by comparison with a DMSO-treated control group. All experiments were carried out in triplicate. The antioxidant activity of each test compound was expressed as an IC_{50} value ± S.D. which was calculated by linear regression analysis. FA was used as a positive control and its IC_{50} value was found to be 0.1 ± 0.04 μM. The radical scavenging activities were expressed as the inhibition percentage and were calculated using the formula: radical scavenging activity (%) = $[(A_0 - A_1/A_0) \times 100]$, where A_0 is the absorbance for control (blank, without compound) and A_1 is the absorbance in the presence of compound.

Lipid peroxidation (LP) assay

LP was assessed by the method of Mihara et al. (1980). Procedures involving the animals and their care conformed to institutional guidelines in compliance with national and international laws and guidelines for the use of animals in biomedical research. Animals were fasted 24 h prior to sacrifice and then sacrificed by decapitation under anesthesia. The livers were immediately removed and washed in ice-cold distilled water, then immediately homogenized with a Teflon homogenizer. LP was measured spectrophotometrically by estimation of thiobarbituric acid reactants (TBARS). Amounts of TBARS were expressed in terms of nmol malondialdehyde (MDA/g tissue). A typical optimized assay mixture contained 0.5 mL of liver homogenate, 0.1 mL of Tris-HCl buffer (pH 7.2), 0.05 mL of 0.1 mM ascorbic acid, 0.05 mL of 4 mM $FeCl_2$ and 0.05 mL of various concentrations of FA derivatives or α-tocopherol; this was incubated for 1 h at 37°C. After incubation, 3.0 mL of H_3PO_4 and 1 mL of 0.6% TBA were added and shaken vigorously. The mixture was boiled for 30 min. After cooling the mixture to room temperature, n-butanol was added and vigorously mixed. The n-butanol phase was separated at 3000 rpm for 10 min. The absorbance of the supernatant was read at 532 nm against a blank, which contained all reagents except the liver homogenate.

Microbiology

Escherichia coli ATCC (American Type Culture Collection) 25922, *Klebsiella pneumoniae* RSKK (Refik Saydam Culture Collection) 574, *Staphylococcus aureus* ATCC 29213, *E. faecalis* ATCC 29212, *Candida albicans* ATCC 10231, *C. krusei* ATCC 6258, *C. pa- rapsilosis* ATCC 90028 and clinical isolates of the bacteria were included in the study. Clinical isolates of *E. coli* and *K. pneumoniae* had extended spectrum beta lactamase (ESBL) and methicilline resistant *S. aureus* isolates (MRSA) were resistant to all beta-lactam antibiotics. *E. faecalis* isolates were resistant to vancomycin (VRE).

Standard powders of ampicillin (Mustafa Nevzat Pharma), gentamicin sulphate (Paninkret Chem. Pharm.), ofloxacin (Zhejiang Huangyan East Asia Chemical Co. Ltd.), meropenem (Astra Zeneca), vancomycin (Mayne Pharma), fluconazole (Sigma), keto-

conazole (Sigma), itraconazole (Sigma), 5-fluorocytosin (Sigma) and amphotericin B trihydrate (Riedel de Haen) were dissolved in appropriate solvents recommended by Clinical and Laboratory Standards Institute (CLSI) guidelines (Rex et al., 2008). Stock solutions of the tested compounds were dissolved in DMSO. Stock solutions of the tested compounds and standard drugs were diluted two-fold in microplate wells. All solvents and diluents, pure microorganisms and pure media were used in control wells. All the experiments were done in 3 parallel series.

Bacteria were subcultured in Mueller Hinton Agar (MHA) (Merck) plates and incubated overnight at 37°C and Candida were subcultured in Sabouraud Dextrose Agar (SDA; Merck) plates at 35°C for 24-48 h.

Bacterial susceptibility testing was performed according to the guidelines of CLSI M100-S18 (Wikler et al., 2009). Mueller Hinton Broth (MHB; Merck) was added to each microplate well. The bacterial suspensions used for inoculation were prepared at 10^5 CFU/mL by diluting fresh cultures at McFarland 0.5 density (10^7 CFU/mL). Suspensions of the bacteria at 10^5 CFU/mL concentrations were inoculated with a two-fold diluted solution of the compounds. A 10 μL bacteria inoculum was added to each microplate well. There were 10^4 CFU/mL bacteria in the wells after inoculations. Microplates were incubated at 37°C overnight. After incubation, the lowest concentration of the compounds that completely inhibited macroscopic growth was determined and reported as the minimum inhibitory concentration (MIC).

Fungal susceptibility testing was performed according to the guidelines of CLSI M27-A3 (Rex et al., 2008). Roswell Park Memorial Institute (RPMI)-1640 medium with L-glutamine (Sigma) buffered to pH 7 with 3-(N-morpholino)propanesulfonic acid (MOPS) (Sigma) was added each microplate well. The colonies were suspended in sterile saline and the resulting suspension was adjusted to McFarland 0.5 density (10^6 CFU/mL). A working suspension was prepared by a 1:100 dilution followed by a 1:20 dilution of the stock suspension. A 10 μL of this suspension at 10^3 CFU/mL was inoculated to a two-fold diluted solution of the compounds. Microplates were incubated at 35°C for 24-48 h. After incubation, the lowest concentration of the compounds that completely inhibits macroscopic growth was determined and reported as MICs.

The minimum biofilm eradication concentration (MBEC) Assay™ for high-throughput screening (HTP; Innovotech) was obtained from the manufacturer and the Calgary Biofilm Device Method (Ceri et al., 1999) was performed to determine the MBEC values for ofloxacin, vancomycin and amphotericin-B and the tested compounds against biofilm forms of the strains.

Subcultures of *S. aureus* and *E. faecalis* were grown on Tryptic Soy Agar (TSA; Merck) for 24 h at 37°C and subcultures of the *Candida* strains were grown on SDA for 24-48 h at 35°C. After checking the purity and viability of the cultures, a second subculture was grown on TSA and SDA plates for bacteria and fungi, respectively and used for the susceptibility testing. McFarland-1 standard culture suspension was prepared and a 30-fold dilution of this suspension was used as the inoculum in the assay. Twenty-two milliliters of the inoculum was poured to the trough of the MBEC Plate and then the peg lid was placed onto the trough. The device was placed on a rotary shaker, which was set to 10° of inclination with 3-6 rocks per min. Biofilms were formed on the lid of the device during incubation for 24 h at 37°C and 48 hat 35°C for bacteria and fungi, respectively. At the same conditions and inoculum, a modified microtitre plate test was applied to all strains and biofilm formation was confirmed (Abdi-Ali et al., 2006). After incubation, the peg lid was transferred to a 96-well plate in which dilutions of the antibacterial agents and the compounds were prepared in tryptic soy broth (TSB; Merck) and dilutions of amphothericin-B and the compounds were prepared in RPMI-1640 medium with L-glutamine buffered to pH 7 with MOPS. These plates were also incubated for the appropriate conditions for bacteria and fungi. After incubation, the peg lid was rinsed twice in physiological saline and transferred to another microplate containing neutralizing agents and the recommended recovery medium. The biofilms were then disrupted into this medium by sonication. After sonication, the peg lid was removed from the recovery medium plate and after incubation, lowest concentration of the agents and the compounds that completely inhibited macroscopic growth was determined as the MBECs.

RESULTS

Chemistry

FA (**3**) was synthesized by reaction of vanillin (**1**) with malonic acid (**2**) by a Knoevenagel-type condensation reaction using aniline as catalyst (Xia et al., 2008). After protection of the phenolic hydroxy group by acetylation (Ac-FA, **4**), esterification was carried out by reaction of corresponding acyl chlorides with appropriate phenols to afford esters in moderate to high yields (**5a-e**). The removal of the acetyl group without the cleavage of the ester bond was performed using hydrazine monohydrate at room temperature

(Nomura et al., 2002) to obtain target ester derivatives of FA (**6a-e**). Structure elucidation of the title compounds were verified by spectroscopic techniques including ^{1}H-NMR, IR and HRMS (LC-MS-TOF). All FA analogs were found to have trans (*E*) configuration confirmed by ^{1}H-NMR spectra in that the coupling constants of α-H and β-H on double bonds were 15.6-16.0 Hz. The purity of the compounds were tested by UPLC with UV detection and determined as 97-100% pure before biological testing. The synthetic route to the desired compounds is depicted in Fig. 1.

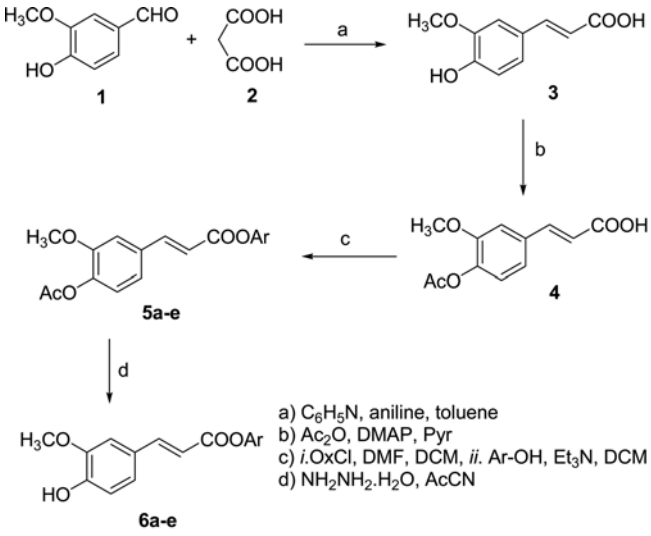


Fig. 1. Synthetic pathways of aromatic ester derivatives of ferulic acid

Antioxidant activity

In the present study, the antioxidant and radical scavenging effects of the synthesized compounds (**5a-e**, **6a-e**) were determined with different in vitro test systems in order to compare the antioxidant potential of compounds for each method. The evaluation of the ester derivatives was carried out at various concentrations with comparison to α-tocopherol, an important antioxidant in living systems. The compounds **5a-e** were OH-protected hydrophobic molecules while the compounds **6a-e** were molecules carrying free phenolic groups.

DPPH free radical scavenging assay

DPPH is a stable free radical and accepts an electron or a hydrogen radical to become a stable diamagnetic molecule and is the most used standard assay in antioxidant activity studies which provides rapid screening for radical scavenging activities of various compounds (Vijay Kumar and Naik, 2010). DPPH free radical scavenging activity of FA and its aromatic ester derivatives with different phenols (**6a-e**) were examined and compared (Table II). FA (**3**) showed appreciable radical scavenging activity at the concentration range of 0.0625 mM to 1 mM. Further coupling of FA with differently substituted phenols (**6a-e**) also resulted in inhibition of DPPH activity although they were not as active as FA at the concentration ranges tested. However, compounds that were OH-protected (**5a-e**) showed weak suppressive activities even at the highest concentrations (1 mM). As seen in Table II, only the aromatic ester derivatives with free phenoxy

Table II. IC_{50} values of various ferulic acid derivatives for superoxide anion radical and DPPH radical scavenging capacities and inhibitory effects on lipid peroxidation[a]

Compounds	Superoxide radical scavenging capacity	DPPH radical scavenging capacity	% Inhibition of lipid peroxidation	
	(IC_{50}, mM)	(IC_{50}, mM)	1 mM	0.1 mM
3 (FA)	NA	0.10 ± 0.04	51 ± 2	10
4	NA	NA	11 ± 4	9
5a	0.61± 0.01	NA	49 ± 2	12
6a	0.43 ± 0.02	0.15 ± 0.05	34 ± 5	25
5b	0.46 ± 0.05	NA	40 ± 2	NA
6b	0.56 ± 0.02	0.13 ± 0.07	25 ± 5	NA
5c	0.57 ± 0.03	NA	35 ± 1	NA
6c	0.55 ± 0.02	0.13 ± 0.02	3 ± 2	NA
5d	0.19 ± 0.04	NA	25 ± 6	NA
6d	0.27 ± 0.01	0.14 ± 0.04	22 ± 3	NA
5e	0.75 ± 0.03	NA	5 ± 1	NA
6e	0.20 ± 0.02	0.14 ± 0.03	2 ± 3	NA
α-tocopherol	0.51 ± 0.05	0.013 ± 0.005	73 ± 2	52 ± 3

NA: Not Active; [a]Each value represents the means ± S.D. of three independent experiments for determination of IC_{50} values.

groups (**6a-e**) and FA itself showed DPPH radical scavenging abilities (IC_{50} values of 0.1-0.15 mM), indicating that the phenolic moiety present in the molecular structure was responsible for the DPPH radical scavenging activity. The presence of hydrophobic branched alkyl groups (**6a-b**), electron withdrawing (**6c**) and electron donating (**6d-e**) substituents on the aromatic ester moiety did not significantly influence the DPPH radical scavenging activity compared to FA.

Superoxide anion radical scavenging assay

The inhibitory effects of FA, Ac-FA and their ester derivatives against $O_2^{\bullet-}$ at different concentrations were tested and calculated IC_{50} values are given in Table II. As shown, FA (**3**) and Ac-FA (**4**) did not show any measurable activity to suppress superoxide generation measured by cytochrome c reduction (inhibition of superoxide generation was 25% for FA and 43% for Ac-FA at 1 mM). However, all tested ester derivatives regardless of the free phenolic group (**5a-e** and **6a-e**) showed significant $O_2^{\bullet-}$-scavenging capacities at concentration ranges between 0.05 and 1 mM and the IC_{50} values were determined from the calibration curve for each compound (Table II). In particular, **5d** (IC_{50} = 0.19 mM), **6d** (IC_{50} = 0.27 mM) and **6e** (IC_{50} = 0.2 mM) had approximately two-fold higher activities as compared to α-tocopherol (IC_{50} = 0.51 mM). All other derivatives also showed comparable IC_{50} values (between 0.43 and 0.75 mM) to α-tocopherol.

LP assay

In the LP process, the initiation of a peroxidation sequence refers to attack of a reactive oxygen species (ROS), such as hydroxyl radical ($OH^{\bullet}$), which easily generates free radicals from polyunsaturated fatty acids. $OH^{\bullet}$ is known as the most efficient ROS to initiate LP (Caliskan-Ergün et al., 2008). In the present study, the LP system forms MDA which can be readily detected by TBA and was used to assess $OH^{\bullet}$ formation. Using the LP assay, FA, Ac-FA and their ester derivatives showed rather weak inhibitory effects on MDA formation at 1 mM and 0.1 mM. Except for compounds **3** (FA) and **5a-c**, no significant activity was observed concerning inhibition of LP (51%, 49%, 40% and 35% at 1 mM, respectively; Table II). These results may suggest that the tested FA derivatives in general might be important as potent superoxide radical scavengers rather than hydroxyl radical scavengers under *in vitro* conditions.

Antimicrobial activity

All the newly synthesized FA derivatives were assayed *in vitro* for antimicrobial activity against several Gram-positive, Gram-negative bacterial strains including drug-resistant isolates and several *Candida* strains. The MIC values were determined by a microdilution method in MHB and RPMI-1640 medium for the antibacterial and antifungal assays, respectively. According to the data (Table III), most of the compounds showed better antibacterial activity against Gram-positive bacteria *S. aureus*, *E. faecalis* and their drug-resistant isolates than other tested Gram-negative bacteria. The results revealed that compounds **6b** and **6c** displayed significant activity against *E. faecalis* with MIC values of 16 μg/mL for each compound. Additionally, these two derivatives had equal to or better activity than gentamycin and vancomycin against vancomycin-resistant *E. faecalis* (MIC for **6b**, 32 and for **6c**, 16 μg/mL). Compound **6c** also demonstrated significant activity against *S. aureus* with a MIC value of <8 μg/mL. All other compounds were moderately active for all tested strains including drug-resistant types. Notably, all of them had better activities than gentamycin against drug-resistant *E. coli* and had comparable activities to gentamycin against drug-resistant *K. Pneumoniae*. On the other hand, compounds **5a-c** and **6c** had noticeable antifungal activities against *Candida* strains (Table III). Compounds **6b** and **6c** had comparable activities with fluconazole for *C. krusei* (MIC values of 64 μg/mL for each compound and fluconazole). Compounds **5a** and **5c** were moderately active against *C. parapsilosis* and *C. albicans*.

Bacterial biofilm eradication activity

In human medicine, drug-resistant nosocomial infections caused by staphylococci have been associated with the growth of these bacteria in biofilms which are known as the most predominant form with high resistance to traditional antibiotics (Dunne, 2002; Vuong and Otto, 2002; Melchior et al., 2006). Once formed, biofilms are difficult to remove as they are 10-1000 times more resistant to antimicrobial agents, when compared with planktonic growing (free floating) bacteria of the same strain (Amorena et al., 1999; Olson et al., 2002). For these reasons, we also tested 8 ester derivatives (**5a-d**, **6a-d**) for their ability to inhibit biofilm formation by *S. aureus* and its isolate, *E. faecalis* and its isolate, *C. krusei* and *C. parapsilosis*. The results are reported as the MBEC along with MIC values for planktonic bacteria in Table III for comparison. Interestingly, compound **6c** caused an inhibition of biofilm formation by *S. aureus* with an MBEC value of <8 μg/mL which was 64 and 128 times more potent than ofloxacin and vancomycin, respectively. We also demonstrated that compounds **5b**, **5c**,

Table III. The antimicrobial, antifungal and antibiofilm activities of the synthesized compounds and control drugs

Compound	Method[a]	Microorganisms										
		Gram-negative				Gram-positive				Fungi		
		Ec	*Ec*[b]	Kp	*Kp*[b]	Sa	*Sa*[b]	Ef	*Ef*[b]	Ca	Ck	Cp
3 (FA)	MIC	256	256	256	256	256	256	128	128	128	128	128
4	MIC	256	256	256	256	256	256	128	128	128	128	128
5a	MIC	128	256	128	256	128	256	128	128	128	128	64
	MBEC	-	-	-	-	512	>1024	1024	1024	-	1024	>1024
6a	MIC	256	256	256	256	256	256	128	128	128	128	128
	MBEC	-	-	-	-	512	>1024	1024	1024	-	1024	>1024
5b	MIC	256	256	256	256	128	128	128	128	128	128	128
	MBEC	-	-	-	-	512	>1024	1024	1024	-	64	>1024
6b	MIC	256	256	128	256	128	128	16	32	64	64	128
	MBEC	-	-	-	-	512	>1024	1024	1024	-	256	>1024
5c	MIC	256	256	256	256	128	256	128	128	64	128	128
	MBEC	-	-	-	-	512	>1024	1024	1024	-	64	>1024
6c	MIC	256	256	128	256	<8	128	16	16	64	64	64
	MBEC	-	-	-	-	<8	>1024	1024	1024	-	64	>1024
5d	MIC	256	256	256	256	256	256	128	128	128	128	128
	MBEC	-	-	-	-	512	>1024	1024	1024	-	64	>1024
6d	MIC	256	256	256	256	256	256	256	128	128	128	128
	MBEC	-	-	-	-	512	>1024	1024	1024	-	64	>1024
5e	MIC	256	256	256	256	256	256	128	128	128	128	128
6e	MIC	256	256	256	256	256	256	128	128	128	128	128
Ampisilin	MIC	2	>2048	2	>2048	0.5	>2048	1	0.5	-	-	-
Gentamisin	MIC	1	1024	0.5	256	0.125	32	4	32	-	-	-
Vankomisin	MIC	-	-	-	-	1	1	1	32	-	-	-
	MBEC	-	-	-	-	>1024	>1024	1024	1024	-	-	-
Ofloksasin	MIC	<0.0625	64	0.125	0.5	0.125	0.25	1	4	-	-	-
	MBEC	-	-	-	-	512	>1024	>1024	1024	-	-	-
Flukonazol	MIC	-	-	-	-	-	-	-	-	1	64	1
AmfoterisinB	MIC	-	-	-	-	-	-	-	-	0.25	2	1
	MBEC	-	-	-	-	-	-	-	-	-	32	32

Abbreviations: *Ec*, *Escherichia coli* ATCC 25922; *Kp*, *Klebsiella pneumoniae* RSKK 574; *Sa*, *Staphylococcus aureus* ATCC 29213; *Ef*, *Enterococcus faecalis* ATCC 29212; *Ca*, *Candida albicans* ATCC 10231; *Ck*, *C. krusei* ATCC 6258; *Cp*, *C. parapsilosis* ATCC 90028.

[a]MIC, Minimal inhibitory concentration (μg/mL); MBEC, minimal biofilm eradication concentraion (μg/mL)

[b]*Ec*, *E. coli* isolate ESBL (resistant to beta lactam antibiotics); *Kp*, *K. pneumoniae* isolate ESBL (resistant to beta lactam antibiotics); *Sa*, *S. aureus* isolate MRSA (resistant to beta lactam antibiotics); *E. faecalis* izolat (VRE) (resistant to vancomycin).

5d, **6c** and **6d** had comparable MBEC values (64 μg/mL) to amphotericin B (32 μg/mL) to inhibit the biofilm formation by *C. krusei* (Table III).

DISCUSSION

Because the main mechanism of action of the phenolic antioxidant, FA, is considered to be the scavenging of free radicals (Graf, 1992), the reactivity of FA derivatives (**5a-e**, **6a-e**) was initially tested toward the stable radical DPPH. Evaluation of antioxidant activity in the DPPH assay with respect to IC_{50} values of the ester derivatives with a free phenolic group seemed to have similar activity. In a homogenous solution, modification of the carboxylic acid side chain of FA by esterification with different phenols does not contribute to the antioxidant potency of FA through intramolecular interactions between the side chain and the phenoxy nucleus. Therefore, the important structural feature for DPPH radical scavenging is the presence of the free phenolic group and not the nature of the aromatic ester chain in the tested compounds.

$O_2^{\bullet-}$ has been implicated in several pathophysiological processes due to its transformation into more reactive species including $OH^{\bullet}$ which initiates LP. $O_2^{\bullet-}$ has also been known to directly initiate LP which significantly contributes to the development of pathological disorders such as atherosclerosis (Halliwell, 1994). In the present study, we demonstrated that all compounds regardless of free phenolic nucleus caused significant attenuation of $O_2^{\bullet-}$ generation. Therefore, we can clearly say that the presence of phenolic group as well as the nature of the aromatic ester chain affects activity since some of the derivatives resulted in potent inhibition of $O_2^{\bullet-}$ (Table II). In particular, the presence of a 4-methoxy-phenyl on the aromatic ester moiety strikingly affected the $O_2^{\bullet-}$ scavenging activity as compared to α-tocopherol. Taken together, all compounds exhibited a scavenging effect on $O_2^{\bullet-}$ generation that could help to prevent or ameliorate oxidative damage. However, with regard to inhibition of LP, none of the compounds demonstrated appreciable activity as demonstrated in rat liver homogenates used as a membrane model (Caliskan-Ergün et al., 2008). Since these differences in antioxidant potency between homogenous and heterogeneous phases were difficult to reconcile, we realized that the latter may be explained by an insufficient anchorage of the side chain with membrane phospholipids. It is very likely that the anchorage of the aromatic ester side chain would negatively affect the positioning of the radical scavenging nucleus of the phenoxy moiety constraining the phenolic nucleus outside of the bilayer surface to quench the flux of the free oxyradicals generated by the Fe^{+3}-ascorbic acid system. This is supported by a previous study where anchoring of molecules within the phospholipids membrane is favored when the side chain on alkyl esters of FA has a high degree of conformational flexibility which enables to bend in a loop conformation toward the ethylenic part of FA (Anselmi et al., 2004). Since our molecules are expected to be fully planar or deviate only marginally from the plane in solution as was shown in a previous study for some FA derivatives (Bakalbassis et al., 2001), it can be speculated that the reported aromatic esters of FA have different anchoring behaviors as opposed to the alkyl esters of FA under the LP assay conditions.

The estimation of MIC has long been the standard for antibiotic susceptibility testing against planktonic organisms and it reveals an important reference for treatment of acute infections. However, potency in terms of MIC values in the treatment of chronic and device-related infections due to bacterial biofilms are often found to be low (Costerton et al., 1995). Many studies have shown that biofilm-grown microorganisms have an inherent lack of susceptibility to antimicrobial agents as compared to planktonic cultures of the same organism (Costerton et al., 1995, 1999; Wilson, 1996; Ceri et al., 1999; Ali et al., 2006). However, this resistance is lost once conditions are reverted to allow planktonic growth (Costerton et al., 1995). In the present study, the biofilm formed by *S. aureus* strain proved to be significantly more resistant to antibiotics than planktonic cultures of the same microorganism (MBECs were 1,000-fold or greater than the MICs for vancomycin and ofloxacin). However, the MBEC value of **6c** fell to within the same range of susceptibility for MIC assay indicating that this compound had a marked effect for eradication of biofilms formed by *S. aureus* as compared to reference antibiotics. This difference in antimicrobial susceptibility between planktonic and biofilm populations may be explained either by differences in the diffusion of **6c** and control antibiotics (Stewart, 1996) or as a result of more complex changes in the microbial physiology of the biofilm (Costerton et al., 1995; Stewart, 1996; Davies et al., 1998); this needs to be investigated further.

In conclusion, we successfully demonstrated that simple aromatic esters of FA (**5d**, **6d-e**) showed significant $O_2^{\bullet-}$ scavenging properties providing useful information for the design of new chemical antioxidant entities with protective activities. Furthermore, antimicrobial evaluation of some of the presented structures (**6b** and **6c**) against Gram-positive bacteria (*S. aureus*, *E. faecalis* and their drug-resistant strains) and fungi (*Candida* strains) are encouraging since compound **6c** also demonstrated potent MBEC values which was comparable to its MIC value for planktonic cultures, and was 64 and 128 times more potent than ofloxacin and vancomycin, respectively. Further studies with these types of compounds are under ongoing investigation in our laboratory.

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