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Synthesis and evaluation of antioxidant and antifungal activities of novel ricinoleate-based lipoconjugates of phenolic acids

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ABSTRACT

Syntheses of four castor oil fatty acid-based novel lipoconjugates of phenolic acids were carried out following Mitsunobu methodology. The lipid part consists of methyl ricinoleate and its saturated analogue, methyl-12-hydroxystearate and the phenolic moieties are ferulic and vanillic acid. Synthesised compounds are evaluated for antioxidant activity using three in vitro assays (DPPH radical scavenging assay, DSC studies for oxidative induction temperature of linoleic acid and autoxidation of linoleic acid in Tween 20 micellar medium) and compared with three widely used antioxidants in the food industry, BHT, α-tocopherol, and dodecyl gallate. Synthesised compounds are found to exhibit good antiradical activity. These compounds also exhibited very good antifungal activity against studied fungal strains. All these results suggested the applicability of the synthesised compounds as potent lipophilic antioxidants for combating oxidative stress.

1. Introduction

Antioxidants are essential to maintain quality of human health as well as quality of food and personal care products. Living organisms are exposed to several environmental oxidative stresses resulting in the generation of a series of oxidants such as peroxides, hydroxyl radical, singlet oxygen species and peroxynitrites. These reactive oxygen species (ROS) are responsible for the oxidative modification of cell membranes, triggering altered cellular mechanisms (Ames, Shigenaga, & Hagen, 1993; Cerutti, 1994; Hussain, Hofseth, & Harris, 2003). Although living organisms are equipped with endogenous antioxidant defence mechanisms, any imbalance in this defence mechanism, especially during ageing, may lead to overproduction of oxidants (Halliwell, 2001). It is necessary to replenish the levels of antioxidants as dietary supplements. Such exogenous antioxidant replenishment is considered a promising approach in the prevention and therapy of diseases caused by oxidants. On the other hand, antioxidants are also important in protecting the nutritive value and increasing the shelf life of cosmetic and food products (Figueroa-Espinoza & Villeneuve, 2005). Lipids in such products, especially those enriched with polyunsaturated fatty acids (PUFAs) are most prone to such oxidative damage, either by autoxidation or by thermal oxidation. For decades, natural antioxidants such as tocopherols, vitamin C and carotenoids have been used as preservatives in the food industry.

Development of natural antioxidants with better antioxidant capacity and less toxicity is desirable for the prevention of diseases and also for improved nutritive value and better shelf life of food products. Natural phenolic compounds, such as flavonoids, polyphenols and phenolic acids, are gaining interest as food preservatives. Phenolics are abundant in the plant kingdom and are one of the most well studied natural antioxidants because of their many potent biological properties (Figueroa-Espinoza & Villeneuve, 2005; Kondratyuk & Pezzuto, 2004). Lipophilisation of natural phenolics can result in novel phenolipids with enhanced antioxidant applications in food/cosmetics and improved bioavailability and bioefficacy under physiological conditions (Shahidi & Zhong, 2010). Ferulic acid, cinnamic acid, sinapic acid, protocatechuic acid, caffeic acid, etc., are some of the phenolics which have been lipophilised with a view to enhance their solubility in apolar media (Buisman et al., 1998; Gaspar et al., 2009, 2010; Guyot et al., 2000; Jose et al., 2011; Kanjilal et al., 2008; Roleira et al., 2010; Sabally, Karboune, Yeboah, & Kermasha, 2005; Sabally, Karboune, St-Louis, & Kermasha, 2006). The result is drastic change in the solubility characteristics of phenolipid without compromising the basic core of the molecule responsible for antioxidant activity. There are many instances where such lipophilisation resulted in an increase of antioxidant activity, compared to the corresponding free phenolic compound (Jose et al., 2011; Reis et al., 2010; Roleira et al., 2010). However, such increase in activity due to lipophilisation depends on the type of medium of the assays (Reddy, Ravinder, Prasad, & Kanjilal, 2011). Moreover, the alkyl chain length of phenolipid was also found to affect profoundly its antioxidant activity

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and a nonlinear dependency of lipophilicity of phenolipid on its antioxidant activity in an emulsion system was reported in the literature (Laguerre et al., 2009, 2010; Reddy et al., 2011). There are also some reports on synthesis of long chain alkyl cinnamic acid esters, where authors studied not only their antioxidant activity but also evaluated their drug-likeness profile (Jose et al., 2011).

In the development of newer antioxidants with potential health benefit for applications in cosmetic and food products, it is necessary to know the physicochemical properties of the molecule, such as hydrophobicity, affinity for the lipid substrate and ability to anchor in the phospholipids bilayer, which governs its antioxidant activity (Figueroa-Espinoza & Villeneuve, 2005). The present work mainly focused on the synthesis, characterisation, and evaluation of four bio-based derivatives designed as lipophilic antioxidants and antifungal agents (Fig. 1). Castor oil contains a unique fatty acid, 12-hydroxy-9-octadecenoic acid (or ricinoleic acid), which is known for its laxative, analgesic and anti-inflammatory effects (Vieira et al., 2000). Two types of phenolics namely vanillic and ferulic acids are esterified with methyl ricinoleate as well as with its saturated analogue, methyl 12-hydroxystearate by the well-known Mitsunobu protocol (Appendino, Minassi, Daddario, Bianchi, & Tron, 2002). Thus the secondary hydroxyl moiety of ricinoleate/12-hydroxystearate is being grafted to the phenolic moiety resulting in a unique class of phenolipid, having a terminal ester moiety and a pendant hydrophobic chain. This type of phenolipid has not been synthesised and studied previously.

The objective of this study is to find the influence of the presence of unsaturation in the alkyl chain and also in the side chain of phenolic acid on the antioxidant activity. The antioxidant activities are studied using three different in vitro assays, namely, DPPH· scavenging assay in polar medium, thermal assay using differential scanning calorimetry (DSC) to measure the oxidative stability of linoleic acid (measured as oxidative induction temperature, OIT), and inhibition of autoxidation of linoleic acid in Tween 20 micellar medium. All the prepared compounds are also evaluated for their antimicrobial activities.

2. Materials and methods

2.1. General

The synthesised phenolic lipids were purified by silica gel (60–120 mesh) column chromatography (Acme Synthetic Chemicals, Mumbai, India) and identified by thin-layer chromatography (TLC), FT-IR, MS, and NMR analysis. TLC was performed on precoated silica gel 60 F_{254} from Merck (Darmstadt, Germany). All ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz (Varian, Palo Alto, CA), respectively. Mass spectra were recorded either on a VG Auto Spec-M (Manchester, UK) or on an Agilent 5973 mass spectrometer (Agilent, Santa Clara, CA) in EI mode. A Lambda-35 UV–Vis spectrophotometer from Perkin Elmer (Waltham, MA) was used in the scavenging assays and also for the estimation of conjugated diene during autoxidation of linoleic acid.

A Perkin Elmer DSC 6000 calorimeter was employed for the thermoxidation stability measurements. The temperature scale was calibrated using In and Zn, and the enthalpy calibration was carried out to the heat of fusion of the same standards. The oxidative stability of pure linoleic acid as well as linoleic acid spiked with antioxidants, measured as oxidative induction temperature (OIT) were analysed by heating samples at 5 K/min at a constant oxygen flow of 50 mL/min. OIT is determined from the plot of heat flow (mW/g) vs. temperature, wherein the exothermic peak indicates the onset of the oxidation process.

2.2. Chemicals

Vanillic acid, ferulic acid and 1,1-diphenyl-2-picryl hydrazine (DPPH) radical were purchased from Fluka (Buchs, Switzerland). Methyl 12-hydroxyoctadec-9-enoate or methyl ricinoleate was prepared from castor oil and purified by column chromatography in the laboratory to get 99% purity. Methyl 12-hydroxystearate was obtained from M/s Jayant Agro-Organics Ltd. (Mumbai, India). Linoleic acid ($\geqslant$99%), α-tocopherol, dodecyl gallate and diisopropylazodicarboxylate (DIAD) were purchased from Sigma–Aldrich (St. Louis, MO). The other chemicals and solvents used were purchased from SD-Fine Chem (Mumbai, India).

2.3. Synthesis

Synthesis of novel lipoconjugates of phenolic acids was carried out by the Mitsunobu protocol (Appendino et al., 2002). Phenolic acid (3 mM) and fatty acid methyl ester (3 mM) were taken in dry tetrahydrofuran (THF, 20 mL) and cooled to 0–4 °C followed by the addition of triphenylphosphine (3 mM) and diisopropylazodicarboxylate (DIAD) (3 mM). The total reaction mixture was stirred at room temperature for 48 h. After 48 h, the organic solvent

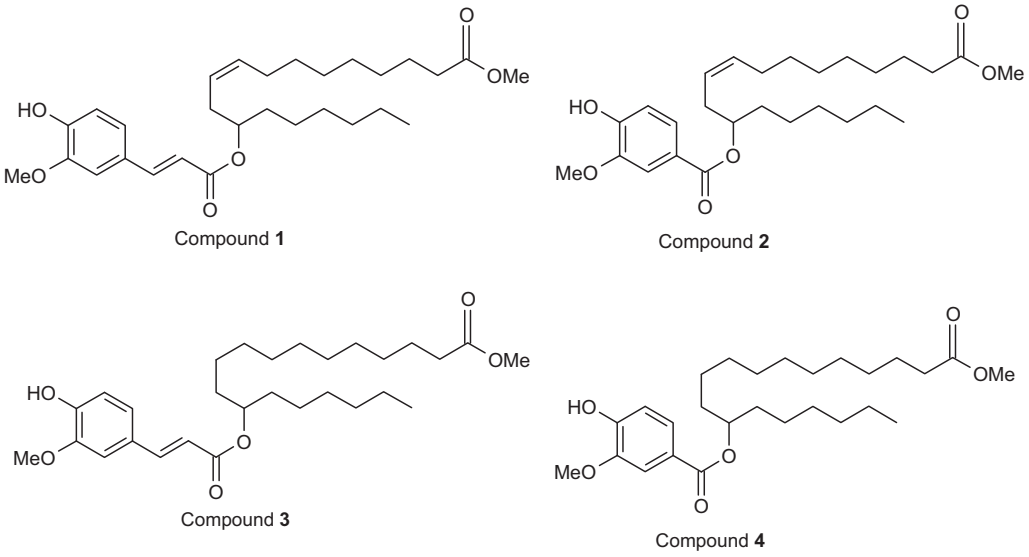


Fig. 1. Structures of the synthesised lipoconjugates of phenolic acids.

was evaporated at 55 °C using a rotary evaporator and the residue was solubilised in ethyl acetate. The organic phase was washed successively with saturated sodium bicarbonate (2 × 50 mL), brine (2 × 50 mL), water (2 × 50 mL), and finally dried over anhydrous sodium sulphate. The organic solvent was evaporated at 55 °C using a rotary evaporator to obtain the crude product, which was purified by column chromatography using hexane/ethyl acetate (90/10; v/v) to get the desired compounds. Spectral characteristic of the four synthesised compounds are given below:

*Methyl-12-(4-hydroxy-3-methoxyphenyl propenoate)octadec-9-en-1-oate (compound **1**):* Light yellow oil (yield 58%); ^{1}H NMR ($CDCl_3$, 200 MHz): δ (ppm): 0.87 (t, 3H, J = 7.39 Hz), 1.28 (m, 16H), 1.54–1.69 (m, 4H), 1.97–2.10 (m, 2H), 2.23–2.42 (m, 4H), 3.66 (s, 3H), 3.92 (s, 3H), 4.93–5.08 (m, 1H), 5.30–5.56 (m, 2H), 5.92 (s, 1H), 6.28 (d, 1H, J = 16.42 Hz), 6.91 (d, 1H, J = 8.21 Hz), 7.02–7.13 (m, 2H), 7.60 (d, 1H, J = 16.42 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ (ppm): 14.0, 22.6, 24.9, 25.4, 27.3, 29.1, 29.2, 29.5, 31.7, 32.0, 33.7, 34.0, 51.4, 55.9, 73.9, 109.2, 114.7, 115.9, 122.9, 124.2, 127.0, 132.6, 144.5146.7, 148.9, 166.9, 174.3; HRMS calculated for $C_{29}H_{44}O_6Na$ $[M+Na]^+$ 511.3035, found 511.3015; EI MS (m/z): 488 ($M^{+\cdot}$).

*Methyl-12-(4-hydroxy-3-methoxybenzoate)octadec-9-en-1-oate (compound **2**):* Light yellow oil (yield, 52%); ^{1}H NMR ($CDCl_3$, 300 MHz): δ (ppm): 0.79 (t, 3H, J = 6.79 Hz), 1.18 (m, 16H), 1.47–1.64 (m, 4H), 1.91–1.99 (m, 2H), 2.22 (t, 2H, J = 7.74 Hz), 2.29–2.43 (m, 2H), 3.59 (s, 3H), 3.87 (s, 3H), 4.98–5.09 (m, 1H), 5.28–5.44 (m, 2H), 6.04 (br s, 1H), 6.85 (d, 1H, J = 8.31 Hz), 7.48 (s, 1H), 7.56 (d, 1H, J = 8.31 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ (ppm): 14.0, 22.5, 24.9, 25.4, 27.3, 29.0, 29.1, 29.5, 31.7, 32.0, 33.7, 34.0, 51.4, 56.0, 74.4, 111.8, 113.9, 122.9, 123.9, 124.2, 132.6, 146.1, 149.8, 166.0, 174.3; HRMS calculated for $C_{27}H_{42}O_6Na$ $[M+Na]^+$ 485.2879, found 485.2860; EI MS (m/z): 462 ($M^{+\cdot}$).

*Methyl-12-(4-hydroxy-3-methoxyphenyl propenoate)octadecan-1-oate (compound **3**):* Light yellow oil (49.2%); ^{1}H NMR ($CDCl_3$, 300 MHz): δ (ppm): 0.88 (t, 3H, J = 7.55 Hz), 1.26 (m, 22H), 1.51–1.62 (m, 6H), 2.26 (t, 2H, J = 7.55 Hz), 3.63 (s, 3H), 3.92 (s, 3H), 4.91–4.99 (m, 1H), 5.92 (br s, 1H), 6.23 (d, 1H, J = 15.86 Hz), 6.87 (d, 1H, J = 8.31 Hz), 6.99–7.08 (m, 2H), 7.54 (d, 1H, J = 15.86 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ (ppm): 13.8, 22.4, 24.7, 25.1, 28.9, 29.0, 29.1, 29.3, 29.5, 31.5, 33.8, 34.1, 51.2, 55.6, 74.1, 109.2, 114.7, 115.6, 122.8, 126.7, 144.4, 146.8, 147.9, 167.0, 174.1. HRMS calculated for $C_{29}H_{46}O_6Na$ $[M+Na]^+$ 513.3192, found 513.3180. EI MS (m/z): 490 ($M^{+\cdot}$).

*Methyl-12-(4-hydroxy-3-methoxybenzoate)octadecan-1-oate (compound **4**):* Light yellow oil (50.8%); ^{1}H NMR ($CDCl_3$, 300 MHz): δ (ppm): 0.85 (t, 3H, J = 6.79 Hz), 1.24 (m, 22H), 1.51–1.65 (m, 6H), 2.24 (t, 2H, J = 7.55 Hz), 3.62 (s, 3H), 3.93 (s, 3H), 4.99–5.08 (m, 1H), 6.13 (br s, 1H), 6.86 (d, 1H, J = 8.31 Hz), 7.49 (s, 1H), 7.57 (d, 1H, J = 8.31 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ (ppm): 14.1, 22.6, 24.9, 25.3, 25.6, 29.1, 29.2, 29.4, 29.5, 29.7, 31.7, 31.9, 33.9, 34.3, 37.5, 51.2, 55.8, 74.3, 111.8, 114.1, 122.6, 123.9, 146.1150.0, 165.7, 173.5; HRMS calculated for $C_{27}H_{44}O_6Na$ $[M+Na]^+$ 487.3035, found 487.3025; EI MS (m/z): 464 ($M^{+\cdot}$).

2.4. Antioxidant activity assays

2.4.1. DPPH radical scavenging assay

The antioxidant activity was determined by the radical-scavenging ability using the stable radical DPPH (Akowuah, Zhari, Norhayati, & Mariam, 2006). Briefly, 200 μL of methanolic solution of the synthesised phenolic lipoconjugates (0.5, 1 and 2 mM) were added to 2 mL of methanolic solution of DPPH radical (0.1 mM) and total volume was made up to 3 mL with methanol. After 40 min incubation at 30 °C in the dark, the absorbance of the mixture was measured at 517 nm against methanol as blank. Ferulic acid, vanillic acid, α-tocopherol, dodecyl gallate and BHT were used as reference and their concentrations were kept the same as that of the synthesised phenolic lipids. The free radical-scavenging activity (FRSA in %) of the tested samples was evaluated by comparison with a control (2 mL of DPPH radical solution and 1 mL of methanol). Each sample was measured in triplicate and averaged. The FRSA was calculated using the formula:

$$\text{FRSA} = [(A_c - A_s)/A_c] \times 100$$

where A_c is the absorbance of the control and A_s is the absorbance of the tested sample after 40 min.

2.4.2. DSC measurements

The antioxidant activity was also evaluated by differential scanning calorimeter (DSC), using pure linoleic acid as a lipid model system (Gaspar et al., 2010; Reis et al., 2010). All the studied antioxidants were taken in methanol to prepare a 1 mM solution. Samples of linoleic acid (2.5–3.0 mg) were placed in standard aluminium pans and spiked with 10 μL of the antioxidant solution. A blank run of linoleic acid, spiked with 10 μL of methanol was also carried out simultaneously to find the oxidative induction temperature (OIT) of linoleic acid. OIT is determined from the first exothermal peak of the plot of heat flow (mW/g) vs. temperature. All the measurements for each antioxidant were run in triplicate.

2.4.3. Autoxidation of linoleic acid in Tween 20 micellar system

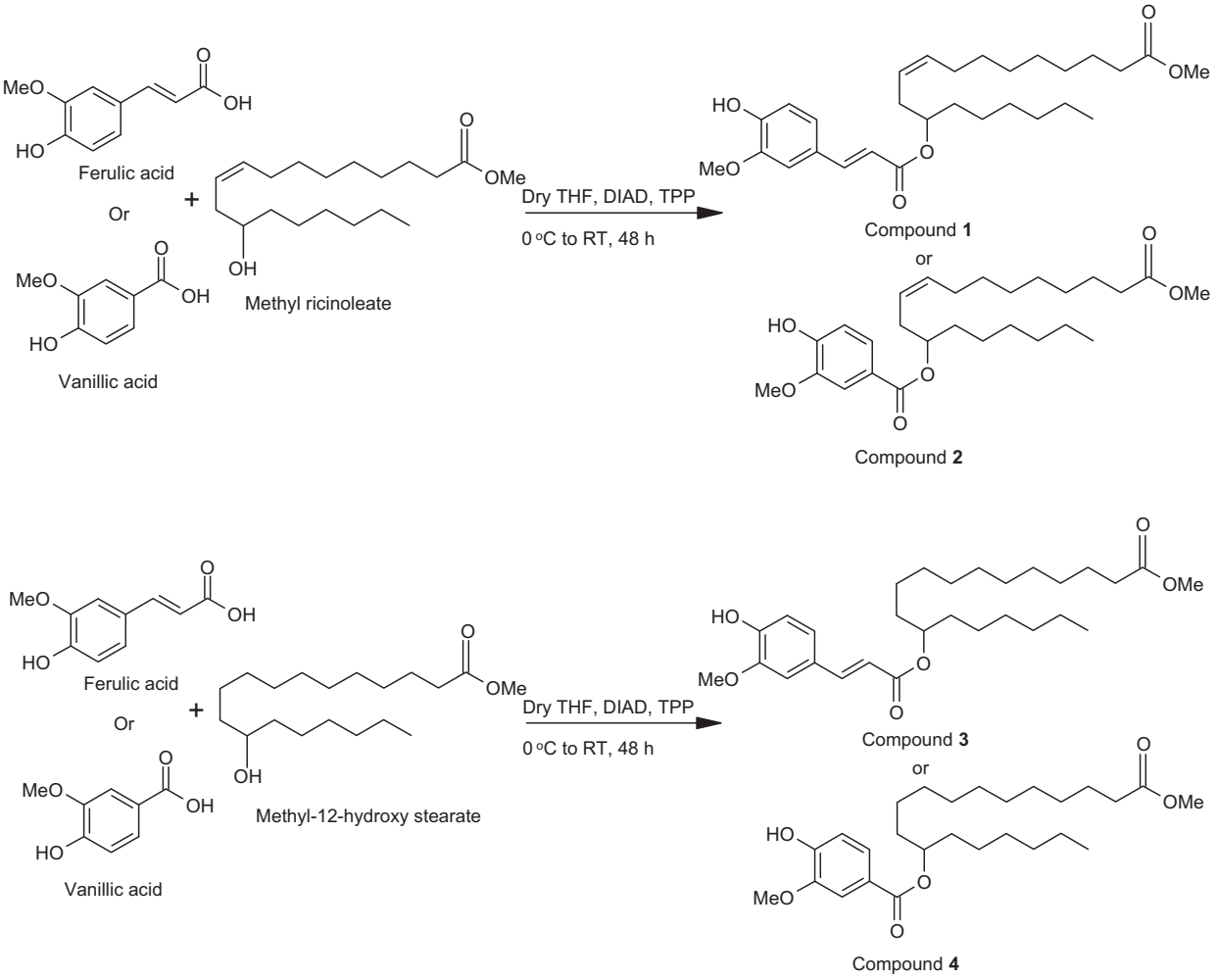
The rate of inhibition of autoxidation of linoleic acid in micelle by antioxidant was measured according to the method of Chimi, Cillard, Cillard, and Rahmani (1991) with some modifications. Linoleic acid (2.5×10^{-3} M) was dispersed with 0.5% Tween 20 in phosphate buffer at pH 6.9 containing 1 mM concentration of reference antioxidants (α-tocopherol, dodecyl gallate and BHT) or synthesised phenolic lipids. Samples were left in the dark and in air for 5 days at 50 °C. Samples without reference and the synthesised antioxidants and the controls without linoleic acid were also incubated under the same conditions. The autoxidation of linoleic acid is accompanied by the generation of conjugated diene, which was measured by UV at 234 nm. Samples were diluted twenty times with phosphate buffer before measuring the absorbance. A decrease in the rate of formation of conjugated diene indicates the increased antioxidant activity of the compound added to the micelle of linoleic acid.

2.5. Antibacterial activity

The minimum inhibitory concentrations (MIC) of synthesised phenolic lipocojugates were tested against three representative Gram-positive organisms, *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 96), *Staphylococcus epidermidis* (MTCC 2639) and Gram-negative organisms *Escherichia coli* (MTCC 443), *Pseudomonas aeruginosa* (MTCC 741), and *Klebsiella pneumoniae* (MTCC 618) by broth dilution method recommended by National Committee for Clinical Laboratory (NCCL, 2000) standards. Standard antibacterial agents like Penicillin and Streptomycin were also screened under identical conditions for comparison.

2.6. Antifungal activity

In vitro antifungal activity of the synthesised phenolic lipocojugates was studied against selected fungal strains namely, *Candida albicans* (MTCC 227), *Candida rugosa* (NCIM 3462), *Saccharomyces cerevisiae* (MTCC 36), *Rhizopus oryzae* (MTCC 262), *Aspergillus niger* (MTCC 282) by agar well diffusion method. The ready-made potato dextrose agar (PDA) medium (Hi-media, 39 g) was suspended in distilled water (1000 mL) and heated to boiling until it dissolved completely. The medium and petri dishes were autoclaved at a pressure of 15 psi (Linday, 1962) for 20 min. The medium was



Scheme 1. Synthetic methodology used for the preparation of castor fatty acid-based lipoconjugates of ferulic and vanillic acids.

Table 1
DPPH radical scavenging activity of the synthesised phenolic lipids.

Compound[a]	Free radical scavenging activity (FRSA)[b] (%)		
	0.5 mM	1.0 mM	2.0 mM
1	60.49 ± 0.67	80.30 ± 0.68	88.45 ± 0.54
2	35.55 ± 1.29	35.00 ± 0.43	36.75 ± 0.81
3	63.35 ± 0.63	81.34 ± 0.52	89.44 ± 0.70
4	33.17 ± 1.40	34.68 ± 0.48	37.41 ± 1.46
FA	76.02 ± 1.53	91.22 ± 1.27	96.13 ± 0.83
VA	45.31 ± 0.54	50.19 ± 0.67	53.45 ± 1.07
ATP	59.38 ± 0.56	78.06 ± 0.64	84.95 ± 1.44
BHT	70.68 ± 0.50	85.93 ± 0.65	88.51 ± 1.33
DDG	75.59 ± 0.91	92.4 ± 1.32	95.78 ± 0.61

[a] **1**: Methyl-12-(4-hydroxy-3-methoxyphenyl propenoate)octadec-9-en-1-oate; **2**: Methyl -12-(4-hydroxy-3-methoxybenzoate)octadec-9-en-1-oate; **3**: Methyl-12-(4-hydroxy-3-methoxyphenyl propenoate)octadecan-1-oate; **4**: Methyl-12-(4-hydroxy-3-methoxy benzoate)octadecan-1-oate; FA: ferulic acid; VA: vanillic acid; ATP: α-tocopherol; BHT: butylated hydroxyl toluene; DDG, dodecyl gallate.
[b] Values are mean ± SD (n = 3).

Table 2
Oxidative induction temperature (OIT) obtained for the pure and inhibited linoleic acid (LA).

Compound[a]	OIT[b] (°C)
LA	112.1 ± 1.27
LA + **1**	162.8 ± 2.05$^{*,\#}$
LA + **2**	157.6 ± 1.47$^{\#}$
LA + **3**	164.5 ± 2.54*
LA + **4**	157.2 ± 0.97$^{\#,*}$
LA + FA	147.8 ± 2.86 *
LA + VA	154.1 ± 1.54$^{\#}$
LA + ATP	156.2 ± 3.72
LA + BHT	162.1 ± 2.79
LA + DDG	160.1 ± 1.07

[a] See Table 1 for abbreviation.
[b] Values are mean ± SD (n = 3), OIT of **1**–**4** is significantly higher than their corresponding free phenolic as shown by *, $p < 0.001$ and #, $p < 0.02$. OIT of **1** is significantly higher than **2**, as shown by #, $p < 0.02$ and that of **2** vs. **4**, as shown by *, $p < 0.001$.

poured into sterile petri dishes under aseptic conditions in a laminar air flow chamber. When the medium in the plates solidified, 0.5 mL of (week old) culture of test organism were inoculated and uniformly spread over the agar surface with a sterile L-shaped rod. Solutions were prepared by dissolving the compound in DMSO and different concentrations were made. After inoculation, wells were scooped out with a 6-mm sterile cork borer and the lids of the dishes were replaced. To each well different concentrations of test solutions were added. Controls were maintained. The treated samples and the controls were kept at 27 °C for 48 h. Inhibition zones were measured and the diameter was calculated in millimetres. Three replicates were maintained for each treatment.

2.7. Statistical analysis

All the assay results reported in the present work are the mean of three measurements (presented as mean ± SD) and were analysed by a paired Student's *t*-test to evaluate the level of statistical significance. A *p*-value less than 0.05 was considered significant.

3. Results and discussion

3.1. Synthesis of novel castor oil based lipoconjugates of phenolic acids

The present work describes the synthesis of four novel lipoconjugates of ferulic and vanillic acids. The novelty is due to the type of fatty acids chosen for lipophilisation, methyl-12-hydroxyoctadec-9-enoate (methyl ricinoleate) and methyl 12-hydroxyoctadecanoate (methyl 12-hydroxystearate). Methyl ricinoleate is the predominant fatty acid present in castor oil and the latter is its saturated analogue. The secondary hydroxyl moiety of these fatty acids is esterified with the phenolic acid by the well-known Mitsunobu esterification methodology (Appendino et al., 2002). The method is simple and chemoselective. Phenolic acids, namely ferulic and vanillic acids were directly esterified with methyl ricinoleate and methyl-12-hydroxystearate in THF in the presence of coupling agent DIAD and triphenyl phosphine to get the corresponding novel phenolic lipoconjugate (Scheme 1); isolated yields are in the range of 49–58%. All these compounds were characterised by NMR and MS. The difference in structure of the synthesised compounds from the other reported phenolic lipids is their connectivity through the secondary hydroxyl moiety positioned at C-12, resulting in a pendant alkyl chain and a terminal ester moiety.

3.2. DPPH radical-scavenging activity

Free radical scavenging is one of the well-known mechanisms through which antioxidants inhibits lipid oxidation. The antioxidant activity of all the synthesised compounds was determined by the radical scavenging ability, using the stable DPPH radical method. This is a standard assay of measuring antioxidant activity and is quite simple and rapid for screening specific compounds. The advantage of this assay is that the DPPH radical is commercially available and does not need to be generated before assay like in other assays. The assay was conducted at three different concentrations of antioxidants (0.5, 1 and 2 mM) in a polar homogeneous medium. The DPPH radical-scavenging capacity of the synthesised compounds is shown in Table 1 along with that of dietary phenolic acids, namely ferulic (FA) and vanillic acid (VA), and three reference antioxidants, namely α-tocopherol (ATP), butylated hydroxyl toluene (BHT) and dodecyl gallate (DDG). Results indicate decreased radical-scavenging activity of lipophilic phenolic compounds compared to the corresponding free phenolic acid. Decrease is significant in the case of compounds **2** and **4** compared to compounds **1** and **3** where marginalisation of scavenging activity in comparison to corresponding free phenolic acid was observed with the increased concentration of antioxidants. This decrease in radical-scavenging activity due to lipophilisation compared to free phenolic acid may be attributed to the increased hydrophobicity of the synthesised phenolipid. Similar views were expressed in recent articles by Laguerre et al. (2010) and Lecomte, Giraldo, Laguerre, Barea, and Villenueve (2010). Another interesting observation is the better radical-scavenging activity of lipoconjugated compounds prepared from ferulic acid compared to those prepared from vanillic acid. This is true even for the free phenolic form. This clearly demonstrates the importance of the allylic double bond of ferulic acid in stabilising the phenoxy radical (Kanjilal et al., 2008).

3.3. DSC measurements

Oxidation of lipid, especially those rich in polyunsaturated fatty acids affects biological systems and is an age-old problem for the food industry. Lipid oxidation reduces not only the nutritive value of the food products but also affects the sensory properties and shelf life (Figueroa-Espinoza & Villeneuve, 2005). Such oxidative modification of lipids requires energy and can be measured by thermal analysis. DSC is one of the most versatile and suitable thermal analytical methods. Using DSC, it is possible to continuously monitor the total thermal effect of lipid oxidation in the microcalorimeter vessel. In the present work, pure linoleic acid was taken as a model lipid system to study the antioxidant activity of the synthesised phenolic lipoconjugates. Autoxidation of linoleic acid is an exothermic process and is indicated by the exothermal peak appearing in the DSC curve, the plot of heat flow vs. temperature (Gaspar et al., 2010; Reis et al., 2010). This peak corresponds to the onset of non-isothermal oxidation of linoleic acid and is measured as oxidative induction temperature (OIT). Thus, antioxidant activity of the synthesised phenolic lipoconjugates was determined

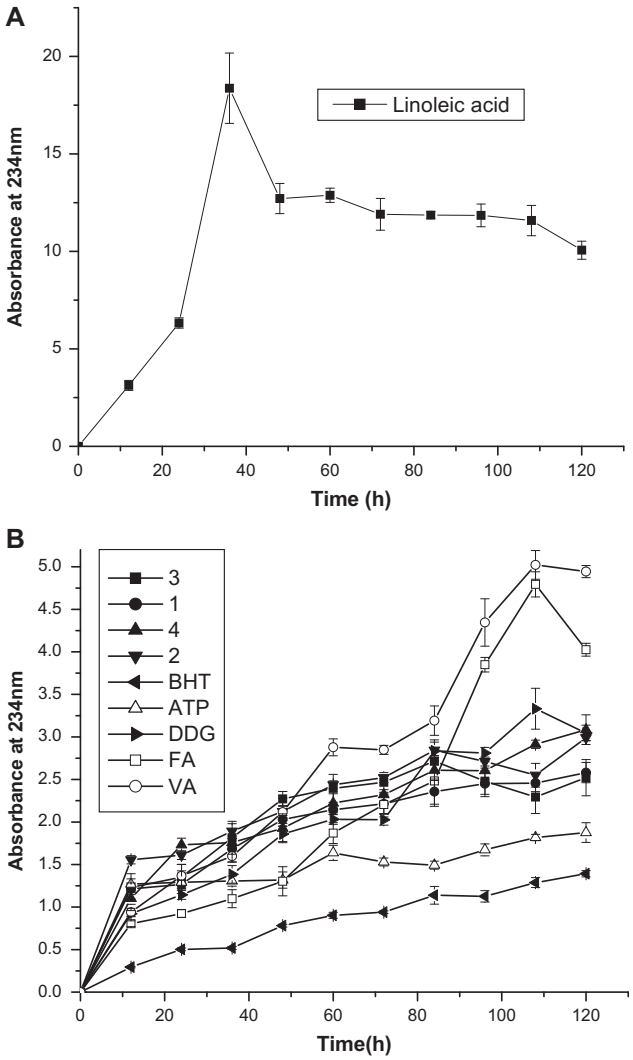


Fig. 2. Spectrophotometric determination of conjugated diene during the 120 h autoxidation of linoleic acid (2.5×10^{-3} M) micelles with the reference antioxidant and the synthesised phenolic lipids (1 mM): (A) linoleic acid and (B) linoleic acid + **1**, ●-●; linoleic acid + **2**, ▼-▼; linoleic acid + **3**, ■-■; linoleic acid + **4**, ▲-▲; linoleic acid + BHT, ◄-◄; linoleic acid + ATP, △-△; linoleic acid + DDG, ►-►; linoleic acid + FA, □-□; linoleic acid + VA, ○-. Compounds **1**–**4**, significantly different from the corresponding free phenolic acids, $p < 0.001$ (see Table 1 for abbreviations).

Table 3
Antifungal activity of the synthesised lipoconjugates of ferulic and vanillic acids.

Compound[a]	Zone of inhibition (mm)									
	C. albicans		*C. rugosa*		*S. cerevisiae*		*R. oryzae*		*A. nizer*	
	100 μg	150 μg	100 μg	150 μg	100 μg	150 μg	100 μg	150 μg	100 μg	150 μg
1	14	19	13	18	12	16	7	10	11	16
2	0	0	6	9	10	14	0	0	10	14
3	9	12	13	19	9	14	7	10	12	18
4	6	9	10	14	6	9	0	0	7	10
Amphotericin B (50 μg)	23.5		22		22		24		25	

[a] See Table 1 for abbreviation.

by measuring the OIT of linoleic acid spiked with free phenolic acids, their corresponding synthesised four lipoconjugates and also three reference antioxidants. Identical DSC curves are obtained for the oxidation of linoleic acid inhibited by these natural and synthetic antioxidants except for the kinetics of linoleic acid oxidation. The results for the oxidative stability of linoleic acid with and without antioxidant, measured as OIT, are shown in Table 2. The OIT of linoleic was found to be 112.1 °C. All the synthesised phenolic lipoconjugates exhibited higher OIT than the control linoleic acid, indicating their ability to penetrate inside the lipid matrix and providing protection to linoleic acid. In fact, activities of all the phenolic lipoconjugates are found to be equal to or marginally better than reference antioxidants. However, ferulic-acid-based lipoconjugates showed significantly higher OIT than vanillic-acid-based lipoconjugates ($p < 0.02$ in the case of compound **1 vs. 2** and $p < 0.001$ in the case of compound **3 vs. 4).** This extra stability is due to the presence of the allylic bond in ferulic acid. The order of reactivity of lipoconjugates is 3 > 1 > 2 = 4. The presence of unsaturation in the hydrophobic part of the ferulic-based lipoconjugate was found to decrease its activity (compound **1**) compared to its saturated analogue (compound **3**). However, no such effect was observed in case of the vanillic-based lipoconjugates (compounds **2** vs. **4**). The activity of lipoconjugates is also found to be enhanced significantly due to lipophilisation when compared to the corresponding native phenolic acids ($p < 0.001$ in the case of compound **1** or **3 vs.** FA and $p < 0.02$ in the case of compound **2** or **4** vs. VA).

3.4. Autoxidation of linoleic acid in Tween 20 micellar system

Antioxidants are widely employed in cosmetic and food products to inhibit lipid peroxidation, which otherwise renders the product rancid and decreases the shelf life and nutritional quality of the product. Most of these products exist in a formulated complex emulsion system. The efficacy of an antioxidant in such a matrix depends not only on its chemical reactivity as radical scavenger but also on the orientation of its radical-scavenging nucleus, interaction with other food components, and environmental conditions (Jose et al., 2011). The assay model taken up in the present work is the rate of autoxidation of linoleic acid in Tween 20 micelle, conducted in the presence of underivatised phenolic compounds and their synthesised lipoconjugates. The effective association of phenolipid with Tween 20 in a mixed micellar form is an important aspect of this assay to inhibit lipid oxidation more efficiently (Laguerre et al., 2009). Initially the auto-oxidation of micelles of linoleic acid is accompanied by a rapid increase of conjugated diene, which is measured at 234 nm. In the control sample, the formation of conjugated diene was found to reach a maximum in 36 h (Fig. 2). The autoxidation of linoleic acid was found to be markedly inhibited ($p < 0.001$), due to the addition of reference antioxidants as well as synthesised lipoconjugates of phenolic acids. This is evident from the negligible formation of conjugated dienes at 36 h. After 120 h of incubation, the inhibition of conjugated diene formation by the lipoconjugates of the phenolics is found to be significantly more ($p < 0.001$) than ferulic and vanillic acids. This indicates appropriate orientation of the reference as well as synthesised antioxidant at the micellar interface compared to free phenolic acids. The order of inhibitions of autoxidation of linoleic acid by the lipoconjugates of phenolic acids (compounds **1**–**4**) is found to be the same as that observed in the DSC study and are similar in value to DDG, the well-known food grade antioxidant. In general, presence of unsaturation at the hydrophobic part of a surfactant makes it a worse candidate for mixed micelle formation with Tween 20, compared to its saturated counterpart. This is somewhat evident in the case of ferulic-based lipoconjugates (**1** vs. **3**), but not significantly so. No such differences were observed in the case of vanillic-based lipoconjugates (**2** vs. **4**). This indicates the ease of mixed micelle formation of these synthesised phenolipids with Tween 20, irrespective of the presence of pendant hydrophobic chain and unsaturation at the hydrophobic part. Thus all the studied antioxidants are appropriately orientated at the micellar interface compared to free phenolic acids. After 120 h of incubation, the order of inhibitions of autoxidation of linoleic acid by all the studied antioxidants is BHT > ATP > DDG = 3 > 1 > 2 = 4 > FA > VA.

3.5. Antifungal activity

Fatty acids and their derivatives may increase the antimicrobial activity of certain organic molecules (Rauf, Gangal, & Sharma, 2008). But none of the synthesised phenolic lipoconjugates showed any activity against the studied bacteria (data not shown). However, compounds **1**–**4** exhibited moderate to good antifungal activities against the tested fungal strains. There were some exceptions, e.g., compound **2** did not show any activity against *C. albicans* and *R. oryzae* (Table 3). Compounds **1** and **3** showed better antifungal activity against all fungal strains compared to compounds **2** and **4**. The reason may be the side-chain double bond present in compounds **1** and **3**.

4. Conclusions

In summary, four novel lipoconjugates of dietary phenolic acids were prepared, where the lipid part is derived from castor oil. The lipid part, ricinoleic acid methyl ester and 12-hydroxystearic acid methyl ester is connected to the phenolic moiety, namely ferulic acid and vanillic acids by chemoselective Mitsunobu esterification. The antioxidant activity of the four novel phenolic esters was studied using DPPH, DSC and autoxidation of linoleic acid in micellar medium. The prepared compounds have shown good antioxidant activity as well as interesting antifungal properties. These novel dietary phenolic lipoconjugates have the advantage of being synthesised from castor oil derivatives, while providing a value-added use of this oil. In conclusion, all these four novel lipoconjugates could be used as potential antioxidants for the storage of lipids and also to combat oxidative stresses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2012.04.046.

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