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Microwave (MW) promoted high yield expedient synthesis of steryl ferulates – A class of novel biologically active compounds: A comparative study of their antioxidant activity with that of naturally occurring γ-oryzanol

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ABSTRACT

Synthetic steryl ferulates [3-O-(*trans*-4-feruloyl)-sterols] are currently gaining considerable importance in recent years to be used as nutraceuticals and food additives as well as in pharmaceutical applications substituting γ-oryzanol – a class of naturally occurring steryl ferulates having potent antioxidant and other organoleptic properties. Considering the importance of this class of compounds coupled with green technology associated with microwave energy (MW) in organic synthesis, we report here an expedited and high yield synthesis of steryl ferulates from abundant steroids, viz., cholesterol, cholestanol, stigmasterol, stigmastanol, β-sitosterol, β-campesterol, β-campestanol and ergosterol applying MW energy in the crucial step of esterification process of sterols with *trans*-4-O-acetylferulic acid to furnish their esterified products, viz., 3-O-(*trans*-4-O-acetylferuloyl)-sterols for their eventual deprotection to their respective steryl ferulates. We further report an efficient and scalable process of producing acetylferulic acid. Testing of synthesized steryl ferulates against antioxidant assays has also been highlighted.

1. Introduction

Antioxidant compounds are currently gaining considerable importance in the field of nutraceutical and food supplement area. Dietary antioxidants are significant as they help in mitigating the adverse effects of oxidative stress caused by an imbalance between reactive oxygen species (ROS) and antioxidant defense system of the body. Oxidative stress causes oxidation of cellular components, like DNA, protein and lipids, resulting in cell damage, which thereby initiates the pathogenesis of several diseases, e.g., diabetes, cardiovascular and neurodegenerative diseases [1]. Besides, endogenous antioxidant systems, exogenous antioxidants such as dietary antioxidants have demonstrated profound health benefit [1]. Currently, many antioxidants, e.g., carotenoids, polyphenols, vitamin E, ascorbic acid, lipoic acid, are used as nutraceuticals [2].

Many naturally occurring compounds act as antioxidants because of their structural characteristics. Ferulic acid is a common antioxidant usually found in foods, its antioxidant property is due to resonance stabilization of its phenoxy radical (**1**) over the phenolic nucleus and the unsaturated side chain [3]. However the antioxidant activity of ferulic acid is decreased in oils at higher temperatures due to limited solubility. Hence esterification of the ferulic acid with sterol provides better solubility in oils at high temperatures [4].

Steryl ferulates can be mostly found in the outer layer of seed kernels, and therefore contribute to the beneficial effects of whole grains. Steryl ferulates thus combine in themselves the health promoting properties of plant sterols and ferulic acid. The key step of their mechanism of action might be their hydrolysis in the gut or gastrointestinal tract. After ingestion, they are supposed to be hydrolyzed into ferulic acid and a free sterol part, which are responsible for their eventual antioxidant and cholesterol-lowering properties [5]. Steryl ferulates are abundant in cereal bran layers and have been identified in rice, wheat, rye, corn and triticale [6]. Among these, the highest level of steryl ferulates has been found in rice (*Oryza sativa* L.), as γ-oryzanol. Its content in rice bran oil is 1.7–2.1% [7]. Chemically, γ-oryzanol is a mixture of steryl ferulates, of which the most commonly occurring are: 24-methylenecycloartanyl ferulate (**2**), cycloartenyl ferulate (**3**), campesteryl ferulate (**4**), campestanyl ferulate (**5**), sitosteryl ferulate (**6**) and sitostanyl ferulate (**7**) [8]. γ-Oryzanol has been shown to be effective against cancer [9], hyperlipidemia and atherosclerosis [10], as well as in lowering of plasma cholesterol [11]. Anticancer

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activity of γ-oryzanol was studied by Klongpityapong et al., showing significant inhibition of cell growth in a dose and time-dependent fashion on human prostate cancer cell lines, DU145 and PC3, as determined by proliferation assay using 3-(4,5-dimethylthiazol,2-yl)-2,5-diphenyl tetrazolium bromide (MTT) [9]. It is also used in sunscreen formulations, nutraceuticals and pharmaceuticals [12].

There are various structural variations of steryl ferulates owing to compositional differences of sterol moieties. These variations have been found to affect antioxidant activity of the steryl ferulates [13]. Furthermore the desmethylsterol esters could exhibit better cholesterol lowering effect than the dimethylsterol esters [14]. Recently it has been demonstrated that the ferulates connected to a saturated sterol exhibited the best antioxidant activity followed by Δ^5-sterols in their experiment as frying oil antioxidants [15]. The same authors further demonstrated that a dimethyl group at C-4 as well as a cyclopropane ring at C-9, C-19 as found in naturally occurring γ-oryzanol, have been found to affect antioxidant activity negatively in frying oils [15]. In view of this, synthesis of steryl ferulates is presently gaining considerable importance for their commercial exploitation to be used as potential substitutes for γ-oryzanol in the area of nutraceutical or food supplements [15]. Besides, synthesis of ferulate esters with non steroidal alcohols too is an important area of research [16,17]. The enzymatic *trans*-esterification of ethyl ferulate with triolein to produce feruloyl-substituted acylglycerols have potential use as sunscreen ingredients [16]. Ethyl ferulate has also been reported as a lipophilic polyphenol, which induces HO-1 protein expression, thereby protecting brain cells against oxidative stress in rats [17]. In general, synthesis of steryl ferulates from sterols and ferulic acid (**8**) comprises three steps: (i) acetylation of *trans*-ferulic acid to its 4-OH protected derivative, *trans*-4-O-acetylferulic acid (ii) esterification of *trans*-4-O-acetylferulic acid with sterols to furnish 3-O-(*trans*-4-O-acetylferuloyl)-sterols, and (iii) deprotection of acetoxy group of 3-O-(*trans*-4-O-acetylferuloyl)-sterols to the desired steryl ferulates, *viz*., 3-O-(*trans*-4-feruloyl)-sterols. Most of the methods so far reported have been found to use pyridine and stringent reaction conditions giving poor to moderate yield of the final product [18,19].

In continuation of our work on the application of microwave energy in steroid transformations [20], we report here a microwave (MW) irradiated expedited high yield synthesis of steryl ferulates from abundant steroids, *viz*., cholesterol (**10**), cholestanol (**11**), stigmasterol (**12**), stigmastanol (**13**), β-sitosterol (**14**), β-campesterol (**15**), β-campestanol (**16**) and ergosterol (**17**), all of which are devoid of dimethyl group at C-4 as well as the cyclopropane ring at C-9, C-19 for their commercial exploitation as better antioxidants. MW energy has been applied in the crucial step of esterification [(step (ii)] for a smooth and fast reaction to provide the esterified products in very high yield. We further report here the application of TMSCl-NaI-Ac_2O or MgI_2-ethereate-Ac_2O as potential acylating systems in the first step of acetylation for protection of 4-OH group of *trans*-ferulic acid (**8**) to produce *trans*-4-O-acetylferulic acid (**9**) in quantitative yield, by eliminating reagents and catalysts like pyridine and mono ammonium salt of 12-tungstophosphoric acid etc. Improvement in both of these steps could result an efficient process for production of steryl ferulates in very high yield in much simplified way and so would have much advantage over the existing methods. We also report a comparative study of the antioxidant activity of the steryl ferulates (**10b–17b**) synthesized with that of naturally occurring γ-oryzanol through (i) radical scavenging activity, (ii) total antioxidant capacity, and (iii) reducing power assay, showing comparable antioxidant activity, thereby exhibiting scope for their commercial exploitations.

2. Experimental

2.1. General methods

Melting points were measured with a Buchi B-540 melting point apparatus and are uncorrected. All the chemicals used were of reagent grade of E. Merck and were used without further purification. IR spectra were recorded with a Perkin-Elmer model 2000 series FT-IR spectrometer for solutions in chloroform. Infrared absorbance is reported in reciprocal centimeters (cm^{-1}). ^1H and ^{13}C NMR spectra were recorded on a Bruker DPX (300 MHz) spectrometer using $CDCl_3$ as solvent with tetramethylsilane (TMS) as internal standard on ppm scale (δ). Multiplicity of the resonance peaks are indicated as singlet (s), broad singlet (bs), doublet (d), triplet (t), quartet (q) and multiplet (m). Mass spectrometric analysis was performed by positive mode electro spray ionization with Bruker Esquire 3000 LC–MS instrument. Elemental analysis was carried out in Varian CHN analyzer. γ-Oryzanol was procured from TCI Chemicals (India) Pvt. Ltd.

2.2. Microwave instrumentation

All MW reactions were carried out in a Synthos 3000 (Anton Paar) microwave reactor. The multitude microwave has a twin magnetron (2.45 GHz) with maximum output power of 1400 W. The output power can be controlled in unpulsed control mode over whole power range which is adjustable in 1 W increment. A Motorola 68xxx series microprocessor system control is used to measure temperature, pressure, time and power during the reaction. The temperature and pressure were monitored throughout the reaction by an IR detector. The temperature can be measured from 0 to 280 °C with uncertainty ±1%. The pressure can be measured from 0 to 86 bar with uncertainty ±0.2 bar.

2.3. Acetylation of ferulic acid (*8*) to trans-4-O-acetylferulic acid (*9*) [*Scheme 1*]

2.3.1. With TMSCl-NaI-Ac_2O system

To a solution of 1.0 g of *trans*-ferulic acid (**8**) (5.1 mmol) in acetic anhydride (2 ml) was added 1 ml of chlorotrimethylsilane [21] followed by 50 mg of sodium iodide. The reaction mixture was then stirred for 20 min at room temperature and was followed on TLC. On completion of the reaction, the reaction mixture was poured into 200 ml of cold water and quenched with aqueous sodium thiosulfate solution to destroy the liberated iodine. It was then extracted with dichloromethane. The organic extract after drying over anhydrous sodium sulfate was evaporated under reduced pressure to get a crude product which was purified through crystallization from alcohol to get pure *trans*-4-O-acetylferulic acid (**9**). The melting point and spectroscopic data of the compound have been directly compared with the literature values [22].

2.3.2. With MgI_2-ethereate-Ac_2O system

To a solution of 1.0 g of *trans*-ferulic acid (5.1 mmol) in dry diethyl ether (10 ml) and acetic anhydride (2 ml) was added a freshly prepared colorless solution of magnesium iodide [23] in dry diethyl ether (1 cm^3, 0.5 mmol) and was heated under reflux for a period of 20 min. After completion of the reaction (TLC), the reaction mixture was quenched with cold water and extracted with hexane. The organic extract after drying over anhydrous sodium sulfate was evaporated under reduced pressure to get a crude product which was purified through crystallization from alcohol to get *trans*-4-O-acetylferulic acid (**9**). The melting point

and spectroscopic data of the compound have been directly compared with the literature values [22].

2.4. MW irradiated esterification of trans-4-O-acetylferulic acid (**9**) with sterol to 3-O-(trans-4-O-acetylferuloyl)-sterol (**10a–17a**)

A mixture of sterol (2.6 mmol), pure *trans*-4-O-acetylferulic acid (**9**) (3 mmol), DCC (2.6 mmol), and DMAP (0.3 mmol) in CH_2Cl_2 (50 ml) was irradiated in a closed vessel in a Synthos 3000 microwave reactor at 400 W, 100 °C and 18 bar for 15 min. The reaction mixture was then allowed to cool to room temperature and dicyclohexylurea formed during the reaction was filtered out. The filtrate was poured into cold water (200 ml) and was extracted with dichloromethane. The organic extract after drying over anhydrous sodium sulfate was evaporated under reduced pressure to get a solid residue of 3-O-(*trans*-4-O-acetylferuloyl)-sterols which was purified through crystallization from alcohol to get pure 3-O-(*trans*-4-O-acetylferuloyl)-sterol as solid.

2.5. Hydrolysis of 3-O-(trans-4-O-acetylferuloyl)-sterols (**10a–17a**) to 3-O-(trans-4-feruloyl)-sterol (steryl ferulates) (**10b–17b**)

To a solution of 3-O-(*trans*-4-O-acetylferuloyl)-sterol (1.75 mmol) in chloroform–methanol (2:1, 50 ml), potassium carbonate (0.30 mmol) was added and refluxed for 6 h [18]. On completion of the reaction, the reaction mixture was quenched with saturated aqueous solution of ammonium chloride and washed with water. The organic extract after drying over anhydrous sodium sulfate was evaporated to dryness to get a crude product which was purified through crystallization from alcohol get pure 3-O-(*trans*-4-feruloyl)-sterol (steryl ferulate) as white solid.

***trans*-4-O-acetylferulic acid (9)**: 1 g of ferulic acid (**8**) was reacted with TMSCl-NaI-Ac_2O or MgI_2-ethereate-Ac_2O as per procedure described above to furnish pure *trans*-4-O-acetylferulic acid (**9**) as white solid (1.14–1.17 g, 94–96%); m.p. 197–201 °C (from EtOH) (lit. [22], 199–201 °C); ^1H and ^{13}C NMR data were found to be consistent with literature values [22]. MS (*m*/*z*): 237 $[M+H]^+$. Anal. calcd. for: $C_{12}H_{22}O_5$: C, 61.02; H, 5.08. Found: C, 61.01; H, 5.06.

3-O-(*trans*-4-O-acetylferuloyl)-cholesterol (10a): 1 g of cholesterol (**10**) was esterified with *trans*-4-O-acetylferulic acid (**9**) under MW irradiation as per procedure described above to get pure 3-O-(*trans*-4-O-acetylferuloyl)-cholesterol (**10a**) as white solid (1.5 g, 95%); m.p. 166–169 °C (from EtOH) (lit. [15], 167–168 °C); IR, ^1H and ^{13}C NMR data were found to be consistent with literature values [15]. MS (*m*/*z*): 627 $[M+Na]^+$. Anal. calcd. for: $C_{39}H_{56}O_5$: C, 77.48; H, 9.27. Found: C, 77.47; H, 9.26.

Cholesteryl ferulate, *viz.*, 3-O-(*trans*-4-feruloyl)-cholesterol (10b): 1 g of 3-O-(*trans*-4-O- acetylferuloyl)-cholesterol (**10a**) was hydrolyzed under basic condition as per procedure described above to get pure cholesteryl ferulate (**10b**) as white solid (890 mg, 96%); m.p. 160–163 °C (from EtOH) (lit. [15], 161–162 °C); IR, ^1H and ^{13}C NMR data were found to be consistent with literature values [15]. MS (*m*/*z*): 585 $[M+Na]^+$. Anal. calcd. for: $C_{37}H_{54}O_4$: C, 79.00; H, 9.61. Found: C, 79.01; H, 9.62.

3-O-(*trans*-4-O-acetylferuloyl)-cholestanol (11a): 1 g of cholestanol (**11**) was esterified with *trans*-4-O-acetylferulic acid (**9**) under MW irradiation as per procedure described above to get pure 3-O-(*trans*-4-O-acetylferuloyl)-cholestanol (**11a**) as white solid (1.46 g, 94%); m.p. 148–153 °C (from EtOH) (lit. [15], 149–168 °C); IR, ^1H and ^{13}C NMR data were found to be consistent with literature values [15]. MS (*m*/*z*): 629 $[M+Na]^+$. Anal. calcd. for: $C_{39}H_{58}O_5$: C, 77.23; H, 9.57. Found: C, 77.20; H, 9.53.

Cholestanyl ferulate, *viz.*, 3-O-(*trans*-4-feruloyl)-cholestanol (11b): 1 g of 3-O-(*trans*-4-O-acetylferuloyl)-cholestanol (**11a**) was hydrolyzed under basic condition as per procedure described above to get pure cholestanyl ferulate (**11b**) as white solid (880 mg, 95%); m.p. 130–133 °C (from EtOH) (lit. [15], 132–133 °C); IR, ^1H and ^{13}C NMR data were found to be consistent with literature values [15]. MS (*m*/*z*): 587 $[M+Na]^+$. Anal. calcd. for: $C_{37}H_{56}O_4$: C, 78.72; H, 9.93. Found: C, 78.73; H, 9.96.

3-O-(*trans*-4-O-acetylferuloyl)-stigmasterol (12a): 1 g of stigmasterol (**11**) was esterified with *trans*-4-O-acetylferulic acid (**9**) under MW irradiation as per procedure described above to get pure 3-O-(*trans*-4-O-acetylferuloyl)-stigmasterol (**12a**) as white solid (1.45 g, 95%); m.p. 185–187 °C (from EtOH); IR: ν_{max}/cm^{-1} 2952, 2868, 1763, 1709, 1509, 1465, 1259, 1155, 1122, 771; NMR: δ_H (300 MHz; $CDCl_3$; Me_4Si) 0.8–1.3 (18H, C(18), C(19), C(21), C(26), C(28) and C(29)Me), 2.2 (3H, s, COMe), 3.9 (3H, s, OMe), 4.7 (1H, m, C(3)H), 5.2 (1H, m, C(23)H), 5.4 (1H, m, C(22)H), 5.43 (1H, m, C(5)H), 6.37 (1H, d, *J* = 16 Hz, C(8′)H), 7.62 (1H, d, *J* = 8.8 Hz, C(7′)H) and 6.8–7.1 (3H, m, Ph); δ_C (75 MHz; $CDCl_3$; Me_4Si) 168.8, 166.2, 151.3, 143.7, 141.3, 139.6, 138.3, 133.5, 129.2, 123.2, 122.7, 121.2, 118.9, 111.14, 74.1, 56.7, 55.9, 55.8, 51.2, 50.0, 42.2, 40.5, 39.6, 37.0, 36.6, 36.6, 31.8, 28.9, 27.8, 25.4, 21.24, 21.1, 21.1, 21.0, 20.6, 20.6, 19.3, 19.3, 19.0, 12.2, 12.0; MS (*m*/*z*): 653 $[M+Na]^+$. Anal. calcd. for: $C_{41}H_{58}O_5$: C, 78.10; H, 9.21. Found: C, 78.09; H, 9.20.

Stigmasteryl ferulate, *viz.*, 3-O-(*trans*-4-feruloyl)-stigmasterol (12b): 1 g of 3-O-(*trans*-4-O-acetylferuloyl)-stigmasterol (**12a**) was hydrolyzed under basic condition as per procedure described above to get stigmasteryl ferulate (**12b**) as white solid (890 mg, 96%); m.p. 145–147 °C (from EtOH); IR: ν_{max}/cm^{-1} 3428, 2940, 2868, 1705, 1635, 1596, 1517, 1273, 1175, 1122, 759; NMR: δ_H (300 MHz; $CDCl_3$; Me_4Si) 0.8–1.3 (18H, C(18), C (19), C(21), C(26), C(28) and C(29)Me), 3.9 (3H, s, OMe), 4.7 (1H, m, C(3)H), 5.0 (1H, m, C(23)H), 5.2 (1H, m, C(22)H), 5.4 (1H, m, C (5)H), 6.31 (1H, d, *J* = 16 Hz, C(8′)H), 7.6 (1H, d, *J* = 8.8 Hz, C(7′)H) and 6.8–7.1 (3H, m, Ph); δ_C (75 MHz; $CDCl_3$; Me_4Si) 166.6, 147.8, 146.7, 144.5, 139.7, 138.3, 129.2, 127.1, 123.1, 122.6, 116.1, 114.6, 109.2, 73.9, 56.7, 55.9, 55.8, 51.2, 50.1, 42.2, 40.5, 39.6, 37.0, 36.6, 36.6, 31.93, 28.9, 27.9, 25.4, 24.3, 21.2, 21.2, 21.1, 21.0, 21.0, 19.3, 19.3, 18.9, 12.1; MS (*m*/*z*): 611 $[M+Na]^+$. Anal. calcd. for: $C_{39}H_{56}O_4$: C, 79.59; H, 9.52. Found: C, 79.57; H, 9.50.

3-O-(*trans*-4-O-acetylferuloyl)-stigmastanol (13a): 1 g of stigmastanol (**13**) was esterified with *trans*-4-O-acetylferulic acid (**9**) under MW irradiation as per procedure described above to get pure 3-O-(*trans*-4-O-acetylferuloyl)-stigmastanol (**13a**) as white solid. (1.4 g, 92%); m.p. 161–163 °C (from EtOH) (lit. [15], 163–164 °C); IR, ^1H and ^{13}C NMR data were found to be consistent with literature values [15]. MS (*m*/*z*): 657 $[M+Na]^+$. Anal. calcd. for: $C_{41}H_{62}O_5$: C, 77.60; H, 9.78. Found: C, 77.58; H, 9.75.

Stigmastanyl ferulate, *viz.*, 3-O-(*trans*-4-feruloyl)-stigmastanol (13b): 1 g of 3-O-(*trans*-4-O-acetylferuloyl)-stigmastanol (**13a**) was hydrolyzed under basic condition as per procedure described above to get stigmastanyl ferulate (**13b**) as white solid (880 mg, 94%); m.p. 154–156 °C (from EtOH) (lit. [15], 156–157 °C); IR, ^1H and ^{13}C NMR data were found to be consistent with literature values [15]. MS (*m*/*z*): 615 $[M+Na]^+$. Anal. calcd. for: $C_{39}H_{60}O_4$: C, 79.05; H, 10.14. Found: C, 79.07; H, 10.21.

3-O-(*trans*-4-O-acetylferuloyl)-β-sitosterol (14a): 1 g of β-sitosterol (**14**) was esterified with *trans*-4-O-acetylferulic acid (**9**) under MW irradiation as per procedure described above to get pure 3-O-(*trans*-4-O-acetylferuloyl)-β-sitosterol (**14a**) as white solid (1.44 g, 95%); m.p. 167–170 °C (from EtOH); (lit. [15], 171–172 °C); IR, ^1H and ^{13}C NMR data were found to be consistent with literature values [15]. MS (*m*/*z*): 655 $[M+Na]^+$. Anal. calcd. for: $C_{41}H_{60}O_5$: C, 77.85; H, 9.49. Found: C, 77.82; H, 9.47.

β-Sitosteryl ferulate, *viz.*, 3-O-(*trans*-4-feruloyl)-β-sitosteryl ferulate (14b): 1 g of 3-O-(*trans*-4-O-acetylferuloyl)-β-sitosterol (**14a**) was hydrolyzed under basic condition as per procedure described above to get β-sitosteryl ferulate (**14b**) as white solid

(870 mg, 94%); m.p. 131–135 °C (from EtOH) (lit. [15], 131–132 °C); IR, ^{1}H and ^{13}C NMR data were found to be consistent with literature values [15]. MS (*m*/*z*): 613 [M+Na]$^+$. Anal. calcd. for: $C_{39}H_{58}O_4$: C, 79.32; H, 9.83. Found: C, 79.30; H, 9.81.

3-O-(*trans*-4-O-acetylferuloyl)-β-campesterol (15a): 1 g of β-campesterol (**15**) was esterified with *trans*-4-O-acetylferulic acid (**9**) under MW irradiation as per procedure described above to get pure 3-O-(*trans*-4-O-acetylferuloyl)-β-campesterol (**15a**) as white solid (1.46 g, 95%); m.p. 160–163 °C (from EtOH); IR: ν_{max}/cm^{-1} 3010, 2862, 1765, 1710, 1640, 1510, 1464, 1080, 758; NMR: δ_H (300 MHz; $CDCl_3$; Me_4Si) 0.7–1.3 (18H, C(18), C(19), C (21), C(25), C(27) and C(28)Me), 2.3 (3H, s, COMe), 3.8 (3H, s, OMe), 4.8 (1H, m, C(3)H), 5.4 (1H, m, C(5)H), 6.36 (1H, d, *J* = 16 Hz, C(8′)H), 7.6 (1H, d, *J* = 8.8 Hz, C(7′)H) and 6.8–7.2 (3H, m, Ph); δ_C (75 MHz; $CDCl_3$; Me_4Si) 168.4, 165.4, 152.4, 144.2, 141.9, 140.3, 133.7, 123.7, 122.5, 121.3, 118.7, 111.3, 74.3, 56.8, 56.2, 55.8, 50.4, 45.8, 42.4, 39.9, 38.5, 37.4, 36.9, 36.4, 34.2, 31.9, 31.6, 29.4, 28.5, 28.1, 24.5, 23.6, 21.5, 20.6, 20.6, 19.4, 19.4, 18.8, 17.4, 17.1; MS (*m*/*z*): 641 [M+Na]$^+$. Anal. calcd. for: $C_{40}H_{58}O_5$: C, 77.67; H, 9.39. Found: C, 77.65; H, 9.40.

β-Campesteryl ferulate, *viz.*, 3-O-(*trans*-4-feruloyl)-β-campesterol (15b): 1 g of 3-O-(*trans*-4-O-acetylferuloyl)-β-campesteryl (**15a**) was hydrolyzed under basic condition as per procedure described above to get pure β-campesteryl ferulate (**15b**) as white solid (870 mg, 94%); m.p. 168–171 °C (from EtOH); IR: ν_{max}/cm^{-1} 3020, 2865, 1712, 1645, 1515, 1470, 1085, 760; NMR: δ_H (300 MHz; $CDCl_3$; Me_4Si) 0.7–1.4 (18H, C(18), C(19), C (21), C(25), C(27) and C(28)Me), 3.9 (3H, s, OMe), 4.8 (1H, m, C (3)H), 5.3 (1H, m, C(5)H), 6.36 (1H, d, *J* = 16 Hz, C(8′)H), 7.7 (1H, d, *J* = 8.8 Hz, C(7′)H) and 6.6–7.1 (3H, m, Ph); δ_C (75 MHz; $CDCl_3$; Me_4Si) 165.3, 152.4, 144.4, 141.8, 140.3, 133.5, 123.7, 122.5, 121.4, 118.8, 111.7, 74.3, 56.8, 56.2, 55.9, 50.4, 45.8, 42.5, 39.9, 38.4, 37.2, 36.9, 36.3, 34.0, 31.9, 31.6, 29.2, 28.3, 28.0, 24.3, 23.6, 21.8, 20.4, 20.4, 19.5, 19.5, 18.6, 17.4; MS (*m*/*z*): 599 [M+Na]$^+$. Anal. calcd. for: $C_{38}H_{56}O_4$: C, 79.17; H, 9.72. Found: C, 79.18; H, 9.73.

3-O-(*trans*-4-O-acetylferuloyl)-β-campestanol (16a): 1 g of β-campestanol (**16**) was esterified with *trans*-4-O-acetylferulic acid (**9**) under MW irradiation as per procedure described above to get pure 3-O-(*trans*-4-O-acetylferuloyl)-β-campestanol (**16a**) as white solid (1.48 g, 96%); m.p. 158–162 °C (from EtOH) (lit. [18], 161–162 °C); IR, ^{1}H and ^{13}C NMR data were found to be consistent with literature values [18]. MS (*m*/*z*): 643 [M+Na]$^+$. Anal. calcd. for: $C_{40}H_{60}O_5$: C, 77.41; H, 9.68. Found: C, 77.42; H, 9.70.

β-Campestanyl ferulate, *viz.*, 3-O-(*trans*-4-feruloyl)-β-campestanol (16b): 1 g of 3-O-(*trans*-4-O-acetylferuloyl)-campesterol (**16a**) was hydrolyzed under basic condition as per procedure described above to get pure β-campestanyl ferulate (**16b**) as white solid (872 mg, 94%); m.p. 155–158 °C (from EtOH) (lit. [18], 157 °C); IR, ^{1}H and ^{13}C NMR data were found to be consistent with literature values [18]. MS (*m*/*z*): 601 [M+Na]$^+$. Anal. calcd. for: $C_{38}H_{58}O_4$: C, 78.89; H, 10.03. Found: C, 78.87; H, 10.10.

3-O-(*trans*-4-O-acetylferuloyl)-ergosterol (17a): 1 g of ergosterol (**17**) was esterified with *trans*-4-O-acetylferulic acid (**9**) under MW irradiation as per procedure described above to get pure 3-O-(*trans*-4-O-acetylferuloyl)-ergosterol (**17a**) as white solid (1.46 g, 94%); m.p. 166–169 °C (from EtOH); IR: ν_{max}/cm^{-1} 2955, 2872, 1766, 1706, 1644, 1509, 1464, 1261, 1221, 1184, 758; NMR: δ_H (300 MHz; $CDCl_3$; Me_4Si) 0.7–1.2 (18H, C(18), C(19), C(21), C(25), C(27) and C(28)Me), 2.2 (3H, s, COMe), 3.9 (3H, s, OMe), 4.7 (1H, m, C(3)H), 5.2 (2H, m, C(5)H, C(6)H), 5.4 (1H, d, *J* = 2.5 Hz, C(23) H), 5.7 (2H, m, C(5) and C(6)H), 6.4 (1H, d, *J* = 16 Hz, C(8′)H), 7.7 (1H, d, *J* = 8.8 Hz, C(7′)H) and 6.8–7.1 (3H, m, Ph); δ_C (75 MHz; $CDCl_3$; Me_4Si) 168.8, 166.2, 151.3, 143.8, 143.8, 141.3, 138.5, 135.5, 133.4, 131.9, 123.2, 121.2, 121.2, 118.8, 116.3, 111.1, 73.0, 55.8, 55.7, 54.5, 46.0, 42.8, 40.4, 39.0, 37.9, 37.1, 36.7, 33.1, 28.2, 28.2, 23.0, 21.1, 21.0, 20.6, 20.6, 19.9, 19.9, 19.6, 17.6, 16.2; MS (*m*/*z*): 637 [M+Na]$^+$. Anal. calcd. for: $C_{40}H_{54}O_5$: C, 78.18; H, 8.79. Found: C, 78.16; H, 8.77.

Ergosteryl ferulate, *viz.*, 3-O-(*trans*-4-feruloyl)-ergosterol (17b): 1 g of 3-O-(*trans*-4-O-acetylferuloyl)-ergosterol (**12a**) was hydrolyzed under basic condition as per procedure described above to get pure ergosteryl ferulate (**17b**) as white solid (875 mg, 94%); m.p. 166–169 °C (from EtOH); IR: ν_{max}/cm^{-1} 3395, 2957, 2871, 1703, 1633, 1594, 1514, 1460, 1269, 1159, 758; NMR: δ_H (300 MHz; $CDCl_3$; Me_4Si) 0.7–1.2 (18H, C(18), C (19), C(21), C(25), C(27) and C(28)Me), 3.9 (3H, s, OMe), 4.7 (1H, m, C(3)H), 5.2 (2H, m, C(5)H, C(6)H), 5.3 (1H, d, *J* = 2.4 Hz, C(23) H), 5.6 (1H, d, *J* = 2.5 Hz, C(22)H), 6.39 (1H, d, *J* = 16 Hz, C(8′)H), 7.6 (1H, d, *J* = 8.8 Hz, C(7′)H) and 6.8–7.1 (3H, m, Ph); δ_C (75 MHz; $CDCl_3$; Me_4Si) 166.6, 147.8, 144.6, 144.6, 141.5, 138.6, 135.5, 131.9, 127.0, 123.0, 120.2, 120.2, 116.3, 114.7, 109.2, 72.7, 55.9, 55.7, 54.5, 46.0, 42.8, 40.4, 39.0, 37.9, 37.1, 36.8, 33.0, 28.3, 28.2, 23.0, 21.1, 21.0, 20.5, 20.5, 19.9, 19.9, 19.6, 17.6; MS (*m*/*z*): 595 [M+Na]$^+$. Anal. calcd. for: $C_{38}H_{52}O_4$: C, 79.72; H, 9.09. Found: C, 79.70; H, 9.08.

2.6. Antioxidant activity assays

2.6.1. Radical scavenging activity

The free radical scavenging activity of the samples was measured using the stable 2,2′-diphenyl-picrylhydrazyl radical (DPPH·) as described originally by Blois [24] with slight modifications. Briefly, various concentrations of each of the synthesized steryl ferulates (**10b**–**17b**) and their mixture (equimolar mixture of **10b**–**17b**), and γ-oryzanol in methanol were taken in different test-tubes. The volume was adjusted to 2 ml with methanol. 0.5 ml of freshly prepared methanolic DPPH solution (0.05 mM) was added to the tubes and shaken vigorously. The tubes were kept in the dark for 30 min at room temperature and then the absorbance of the samples was measured at 517 nm using a UV–Vis spectrophotometer (Lambda 35, Perkin-Elmer, Singapore). The free radical scavenging activity of all the compounds was calculated as percentage of DPPH discoloration using the following formula:

$$\% \text{ Radical scavenging activity} = [(A_o - A)/A_o] \times 100$$

where A_o is the absorbance of the solution without the compound, and A is the absorbance of the solution with the compound.

The scavenging activity of each of the compound was expressed as 50% effective concentration, EC_{50} (mg/ml), which refers to the concentration of the compound that produces 50% scavenging of the DPPH· radical.

2.6.2. Total antioxidant capacity

The total antioxidant capacity of the compounds, *viz.*, synthesized steryl ferulates (**10b**–**17b**) and their mixture (equimolar mixture of **10b**–**17b**), and γ-oryzanol, was determined by the Cupric Reducing Antioxidant Capacity (CUPRAC) assay, as described in literature [25]. Briefly, 1 ml each of $CuCl_2$ solution (1.0×10^{-2} M), neocuproine solution (7.5×10^{-3} M) and ammonium acetate buffer (pH 7.0, 1.0 M) were added to a test- tube. After that 0.4 ml sample solution in methanol and 0.7 ml of deionized water were added and mixed. After 30 min, the absorbance was measured at 450 nm using a UV–Vis Spectrophotometer (Lambda 35, Perkin-Elmer, Singapore). The antioxidant capacity was determined against Trolox standard and the results were expressed as μmol Trolox equivalent (TE) per g of compound.

2.6.3. Reducing power assay

The reducing power of the compounds, *viz.*, synthesized steryl ferulates (**10b**–**17b**) and their mixture (equimolar mixture of **10b**–**17b**), and γ-oryzanol, was determined according to the method of Oyaizu [26]. Briefly, different concentrations of the

sample solutions (2.5 ml) in methanol were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Thereafter, 2.5 ml of 10% trichloroacetic acid was added, and the mixture was centrifuged at 3000 rpm for 10 min. 5 ml of the upper layer was mixed with 5 ml deionized water and 1 ml of 0.1% ferric chloride and equilibrated for 30 min. The absorbance was then measured at 700 nm using a UV–Vis spectrophotometer (Lambda 35, Perkin-Elmer, Singapore).

2.7. Statistical analysis

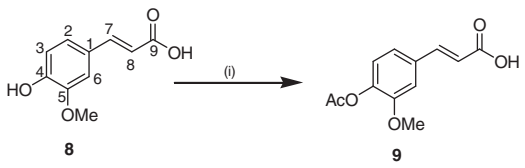
All the antioxidant assays were performed in triplicate and the data were reported as mean ± standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA) using OriginPro (v.8, Northampton, MA, USA). The significant differences ($p < 0.05$) between the means was determined using Tukey test set at $\alpha = 0.05$.

3. Results and discussion

3.1. Synthesis of acetyl ferulic acid and steryl ferulates

Schemes 1 and 2 describe the synthetic approaches carried out in the present work. The first step of acetylation of aromatic hydroxyl group (Scheme 1) of ferulic acid (**8**) is generally done by most of the researchers using acetic anhydride in presence of pyridine [18,19]. However, on trying this reaction, we found that the reaction proceeded very slow, and even after keeping for 1–2 days at room temperature, only 40–50% conversion was observed, while at higher temperature and under microwave condition, decomposition took place giving number of products. In another report, the acetylation of ferulic acid had been carried out by using pyridine and DMAP in the reaction giving very high yield of acetyl ferulic acid [15,22]. However the use of pyridine is restricted because of its toxic and non-environment friendly nature. Some other work involved the use of a heterogeneous catalyst, *viz*., monoammonium salt of 12-tungstophosphoric acid, in presence of acetic anhydride at room temperature to eliminate the use of pyridine in the reaction [19]. They further reported the use of MW energy in this acetylation process to expedite the reaction time with increase in yield of *trans*-4-O-acetylferulic acid.

Another group of researchers reported a chemoenzymatic synthetic approach to get steryl ferulates wherein the first step of protection of phenolic group of ferulic acid was carried out by preparing vinyl ferulate by *lipase* catalyzed *trans*-esterification in presence of vinyl acetate [27]. However, the poor yield (42–46%) of protected ferulic acid (vinyl ferulate) for the subsequent second step of esterification is the main drawback of this method. To overcome all these drawbacks, we report here a simple and convenient but expedited acetylation process for preparing *trans*-4-O-acetylferulic acid (**9**) from ferulic acid (**8**) in almost quantitative yield through *in situ* generation of iodotrimethylsilane using TMSCl-NaI-Ac_2O or by using MgI_2-ethereate-Ac_2O system; both these methods were in earlier occasions reported to be fast acylating systems for primary, secondary, tertiary and aromatic hydroxyl groups as well as for enol acetylation of carbonyl compounds [21,23]. Thus when ferulic acid was reacted with either TMSCl-NaI-Ac_2O or MgI_2-ethereate-Ac_2O system, *trans*-4-O-acetylferulic acid was formed in a very fast and clean reaction (20 min) giving almost quantitative yield (Scheme 1). Hence the application of these cheap and easily available reagents eliminating the use of pyridine would make the procedure quite advantageous and economic over the existing methods in preparing *trans*-4-O-acetylferulic acid (**9**) from ferulic acid (**8**) for its eventual esterification with sterols to be used in food industry.



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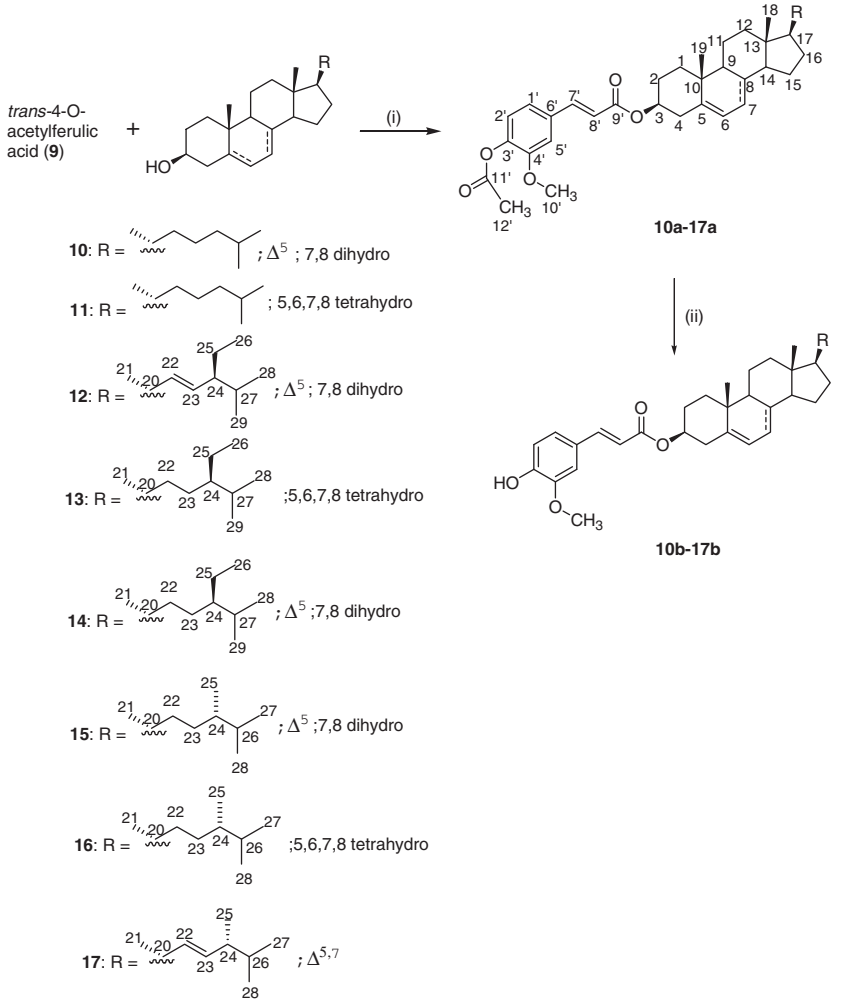
Scheme 1. Reagents and conditions: (i) TMSCl-NaI, Ac_2O, rt, 20 min 94%, or MgI_2-ethereate-Ac_2O, reflux, 20 min, 96%.

Having succeeded in converting ferulic acid to its 4-OH-protected derivative, *viz*., *trans*-4-O-acetylferulic acid (**9**) in such high yield, we aimed at synthesizing a few steryl-4-O-acetylferulates starting from readily available sterols, *viz*., cholesterol (**10**), cholestanol (**11**), stigmasterol (**12**), stigmastanol (**13**), β-sitosterol (**14**), β-campesterol (**15**), β-campestanol (**16**) and ergosterol (**17**), through esterification with *trans*-4-O-acetylferulic acid (**9**) (Scheme 2). As per literature reports, esterification of sterols with *trans*-4-O-acetylferulic acid is generally carried out by refluxing their mixture in presence of DCC (as dehydrating agent) and DMAP (as catalyst) in dichloromethane to get their respective steryl-4-O-acetylferulates for their eventual deprotection to steryl ferulates, *viz*., 3-O-(*trans*-4-feruloyl)-sterols [18,19]. However when we tried to synthesize cholesteryl ferulate, even after refluxing it with *trans*-4-O-acetylferulic acid in DCM with DCC and DMAP for a period of 20 h, only 35–40% conversion was observed leaving majority as the unreacted material. Similar was the case with the other sterols (**11–17**). In a most recent paper, modification of the esterification procedure has been reported by carrying out this esterification reaction at 0 °C followed by at room temperature under nitrogen atmosphere to give 77–90% yield of steryl ferulates within 1.5 h [15].

This led us to hit upon the idea of applying microwave (MW) energy in this esterification process to make it more efficient and expedient. One of the most fundamental obstacles in developing technologies is to minimize the energy consumption and to minimize or eliminate the use of toxic and hazardous substances. In this regard, the application of MW energy to bring about chemical reaction is a suitable alternative, as it takes care of two very necessary criteria of synthesis, *viz*., energy consumption for heating and the time required for the reaction [28]. Therefore, during the last few years, studies on the effect of MW irradiation in organic and macromolecules has become a subject of considerable interest [29]. Accelerating effect and efficient non-contact heating are two important traits of MW irradiated reactions. The accelerating effects of MW irradiation, has recently been demonstrated by Carsten et al. in their work on polymer chemistry [30]. In our recent work we demonstrated the application of MW energy in expediting Baeyer-Villiger oxidation and the synthesis of novel steroid-amino acid conjugates giving high yield in very fast and clean reactions [20]. Thus when a mixture of cholesterol (**10**), *trans*-4-O-acetylferulic acid (**9**), DCC and DMAP in dichloromethane was irradiated with microwave (400 W, 18 bar, 100 °C), the esterification process proceeded rapidly to furnish 3-O-(*trans*-4-O-acetylferuloyl)-cholesterol (**10a**) in 95% yield in a very clean and fast reaction (15 min) without any unreacted material (Scheme 2). Similar was the case with other sterols, *viz*., cholestanol (**11**), stigmasterol (**12**), stigmastanol (**13**), β-sitosterol (**14**), β-campesterol (**15**), β-campestanol (**16**) and ergosterol (**17**), resulting in 90–95% yield of their corresponding 3-O-(*trans*-4-O-acetylferuloyl)-derivatives (**11a–17a**) in fast and clean reactions, with no side products. The method will definitely be advantageous over the existing methods in synthesizing steryl ferulates for their commercial exploitation.

The final step of deprotection was carried out by simple base hydrolysis, *viz*., refluxing 3-O-(*trans*-4-O-acetylferuloyl)-sterols



Scheme 2. Reagents and conditions: (i) DCC, DMAP, CH_2Cl_2, MW, 400 W, 15 min, 95%; (ii) K_2CO_3, $CHCl_3$–MeOH (2:1), reflux, 6 h, 95%.

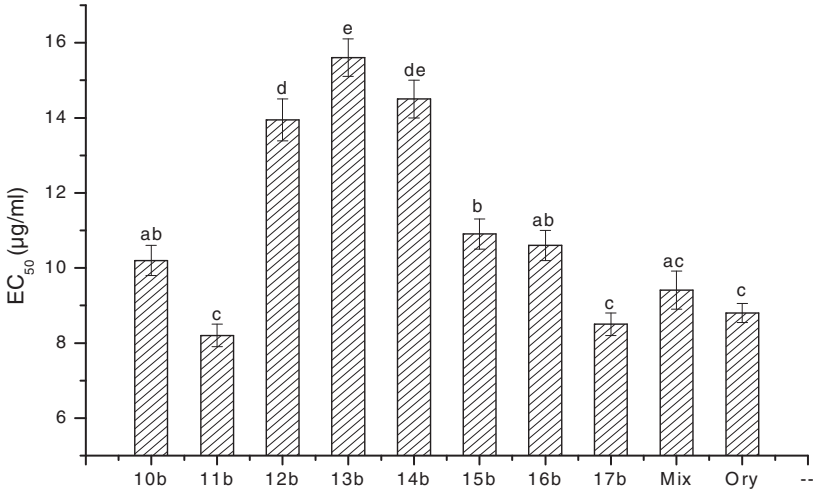


Fig. 1. EC_{50} values of 3-O-(*trans*-4-feruloyl)-cholesterol (**10b**), 3-O-(*trans*-4-feruloyl)-cholestanol (**11b**), 3-O-(*trans*-4-feruloyl)-stigmasterol (**12b**), 3-O-(*trans*-4-feruloyl)-stigmastanol (**13b**), 3-O-(*trans*-4-feruloyl)-sitosterol (**14b**), 3-O-(*trans*-4-feruloyl)-campesterol (**15b**), 3-O-(*trans*-4-feruloyl)-campestanol (**16b**), 3-O-(*trans*-4-feruloyl)-ergosterol (**17b**), mixture of synthesized steryl ferulates (**10b**–**17b**) (**Mix**), and γ-oryzanol (**Ory**), as determined by DPPH radical scavenging assay. Each bar represents the mean ± SD. Bars with the same superscript are not significantly different ($p < 0.05$) from each other.

(**10a**–**17a**) with potassium carbonate in chloroform–methanol (2:1) to get their respective steryl ferulates, *viz*., cholesteryl ferulate, *viz*., 3-O-(*trans*-4-feruloyl)-cholesterol (**10b**), cholestanyl ferulate, *viz*., 3-O-(*trans*-4-feruloyl)-cholestanol (**11b**), stigmasteryl ferulate, *viz*., 3-O-(*trans*-4-feruloyl)-stigmasterol (**12b**), stigmastanyl ferulate, *viz*., 3-O-(*trans*-4-feruloyl)-stigmastanol (**13b**), β-sitosteryl ferulate, *viz*., 3-O-(*trans*-4-feruloyl)-β-sitosterol (**14b**), β-campesteryl ferulate, *viz*., 3-O-(*trans*-4-feruloyl)-β-campesterol

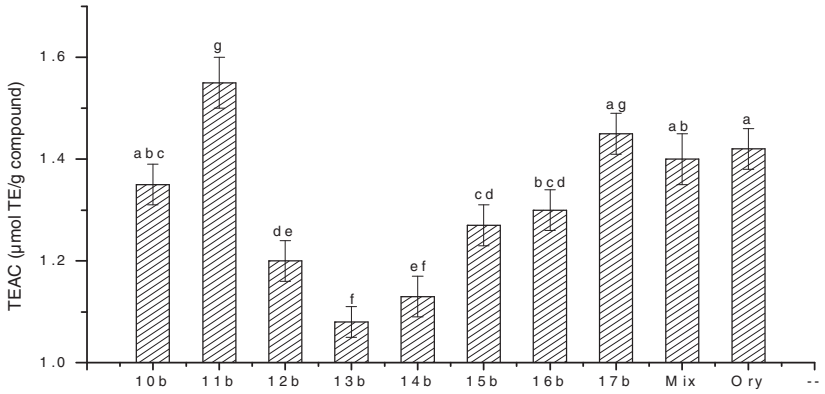


Fig. 2. TEAC values of 3-O-(*trans*-4-feruloyl)-cholesterol (**10b**), 3-O-(*trans*-4-feruloyl)-cholestanol (**11b**), 3-O-(*trans*-4-feruloyl)-stigmasterol (**12b**), 3-O-(*trans*-4-feruloyl)-stigmastanol (**13b**), 3-O-(*trans*-4-feruloyl)-sitosterol (**14b**), 3-O-(*trans*-4-feruloyl)-campesterol (**15b**), 3-O-(*trans*-4-feruloyl)-campestanol (**16b**), 3-O-(*trans*-4-feruloyl)-ergosterol (**17b**), mixture of synthesized steryl ferulates (**10b**–**17b**) (**Mix**), and γ-oryzanol (**Ory**), as determined by CUPRAC assay. Each bar represents the mean ± SD. Bars with the same superscript are not significantly different ($p < 0.05$) from each other.

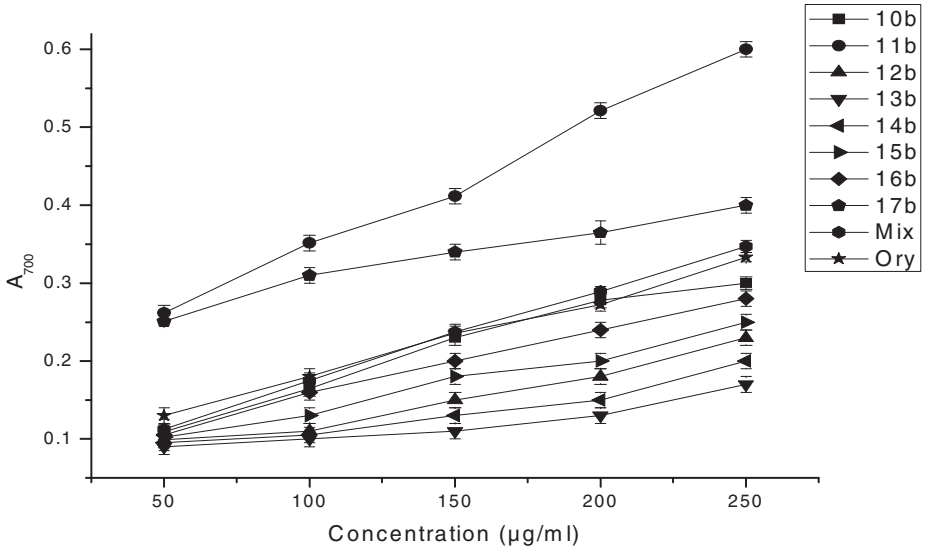


Fig. 3. Reducing power of 3-O-(*trans*-4-feruloyl)-cholesterol (**10b**), 3-O-(*trans*-4-feruloyl)-cholestanol (**11b**), 3-O-(*trans*-4-feruloyl)-stigmasterol (**12b**), 3-O-(*trans*-4-feruloyl)-stigmastanol (**13b**), 3-O-(*trans*-4-feruloyl)-sitosterol (**14b**), 3-O-(*trans*-4-feruloyl)-campesterol (**15b**), 3-O-(*trans*-4-feruloyl)-campestanol (**16b**), 3-O-(*trans*-4-feruloyl)-ergosterol (**17b**), mixture of synthesized steryl ferulates (**10b**–**17b**) (**Mix**), and γ-oryzanol (**Ory**). Each value is expressed as mean ± SD.

(**15b**), β-campestanyl ferulate, *viz.*, 3-O-(*trans*-4-feruloyl)-β-campestanol (**16b**) and ergosteryl ferulate, *viz.*, 3-O-(*trans*-4-feruloyl)-ergosterol (**17b**) in almost quantitative yield (Scheme 2).

3.2. Antioxidant activity of steryl ferulates

Antioxidant activity of synthesized steryl ferulates (**10b**–**17b**) and their mixture thereof (**Mix**), and γ-oryzanol (**Ory**) was determined by the radical scavenging assay (DPPH), total antioxidant capacity assay (CUPRAC) and reducing power assay.

The radical scavenging activity of the synthesized steryl ferulates (**10b**–**17b**) and their mixture (**Mix**) (equimolar mixture of **10b**–**17b**), and γ-oryzanol (**Ory**), was determined using the DPPH free radical. Antioxidants quench DPPH free radicals, converting them to 2,2-diphenyl-1-hydrazine, which results in a decrease in absorbance at 517 nm. The results were expressed as EC_{50} (Fig. 1), which is the amount of antioxidant that reduces the initial DPPH concentration by 50%. The mixture of the synthesized steryl ferulates and γ-oryzanol were better radical scavengers than the individual ferulates, **10b, 12b**–**16b**. The sterol structure of the synthesized steryl ferulates had a distinct effect on their activity, as is evidenced by the varying EC_{50} values of the synthesized steryl ferulates. The mixture of the synthesized steryl ferulates was comparable to the radical scavenging activity of standard γ-oryzanol. Significant difference in the EC_{50} values was observed among the compounds ($p < 0.05$).

The total antioxidant capacity of the synthesized steryl ferulates (**10b**–**17b**) and their mixture (**Mix**) (equimolar mixture of **10b**–**17b**), and γ-oryzanol (**Ory**), as determined by the CUPRAC assay are represented in Fig. 2. The assay involves the use of the chromogenic redox reagent, bis(neocuproine)copper(II) chelate. The antioxidants reduce the bis(neocuproine)copper(II) chloride, (Cu(II)-Nc), to $Cu(Nc)_2^+$ chelate, which shows maximum absorption at 450 nm. The TEAC values of the investigated compounds decreased in the following order: 11b > 17b > Ory > Mix > 10b > 16b > 15b > 12b > 14b > 13b. There was significant difference among the TEAC values of the compounds ($p < 0.05$).

The reducing power of the synthesized steryl ferulates (**10b**–**17b**) and their mixture (Mix) (equimolar mixture of **10b**–**17b**), and γ-oryzanol (Ory), was plotted as a function of their concentration (Fig. 3). The antioxidant (reducing) nature of the compounds reduces the Fe^{3+}/ferricyanide complex to the ferrous (Fe^{2+}) form, which can be monitored by measuring the formation of Prussian blue at 700 nm. The reducing power of the compounds increased

with concentration. Between 100 and 150 μg/ml concentration range, the mixture of the steryl ferulates (**Mix**) and γ-oryzanol exhibited comparable reducing power; while at concentrations above this range, the reducing power of the mixture was higher than γ-oryzanol.

4. Conclusion

In conclusion we describe a three-step synthesis of various steryl ferulates and their testing against antioxidant assays coupled with an efficient and scalable process of producing acetylferulic acid. A fast and efficient process for linking acetylferulic acid to sterols through esterification by the application of Microwave (MW) Energy would have much advantage over the existing methods. Steryl ferulates are found to possess well-established health-promoting properties, among which antioxidant and cholesterol lowering activity are the most extensively studied. The compounds like sterol and stanol fatty acid esters, which are currently added to functional foods to affect cholesterol lowering activity, have similar mechanism of action as steryl ferulates, which release free sterols in the gut as mentioned earlier. Thus the present work highlights the application of synthetic steryl ferulates as potential substitutes for γ-oryzanol in the field of nutraceutical and food additives besides finding use in pharmaceutical applications as well.

Conflict of interests

The authors confirm that the content of this work has no conflict of interests.

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