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RESEARCH ARTICLE

Synthesis, Characterization and Preliminary Cytotoxic Study of Sinapic Acid and its Analogues

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Abstract

The use of natural products to treat human diseases especially cancer is an interesting trend in pharmaceutical research. Sinapic acid, being a member of hydroxycinnamic acid family and having well-established pharmacological properties, could have a potential antitumor activity that has not been investigated sufficiently. This work aimed to test the preliminary cytotoxic effect of sinapic acid and nine of its synthesized analogues utilizing MTT assay on four cancer cell lines; which are: HeLa, AMN3, SKG, and MCF-7. The chemical structures of the intermediates and of the synthesized products were approving by analyzing their IR, ^{1}H-NMR, ^{13}C-NMR, and MS-ESI spectra. Results of the cytotoxicity study indicated that sinapic acid analogues 3a, 3c, 3d, 3e and 3h have lower IC_{50} values than that of positive control on the tested cancer cell lines, while sinapic acid itself and the other synthesized analogues did not have such cytotoxic effect.

Keywords: *Sinapic acid, Analogues, Gastrodigenin, MTT, Cytotoxicity.*

Introduction

Cancer is a foxy disease that can start and progress without any warning. This, combined with the difficulty of its treatment, makes cancer a serious global health problem that can strike anyone at any time [1]. Cancer is a condition in which the cellular monitoring of apoptosis is lost leading to uncontrolled proliferation of cells. This results in the formation of neoplastic tumor that starts benign until its progression to the malignancy as it metastasizes to other tissues or organs [2].Utilization of natural products for management of various human disorders including cancer became an interesting area of research in the last decades [3]. Phytochemicals with antitumor activity can exert their effect through different mechanisms such as induction of apoptosis, inhibition of topoisomerase I or II, and modification of cellular metabolism [4].Phenolic compounds are a group of key secondary metabolites found mainly in the plant kingdom and they have a wide spectrum of physiological activities [5]. One of the most interesting classes of such compounds is a hydroxycinnamic acids family, to which sinapic, p-coumaric, ferulic, vanillic and caffeic acids belong [6]. Sinapic acid (Figure 1) is frequently found in human diet because of its high prevalence in many vegetables (e.g. white onion), fruits (e.g. lemon), cereal grains (e.g. rice) and herbs (e.g. borage). Like other members of its family, sinapic acid is naturally found in the free form or as a glycoside [7].

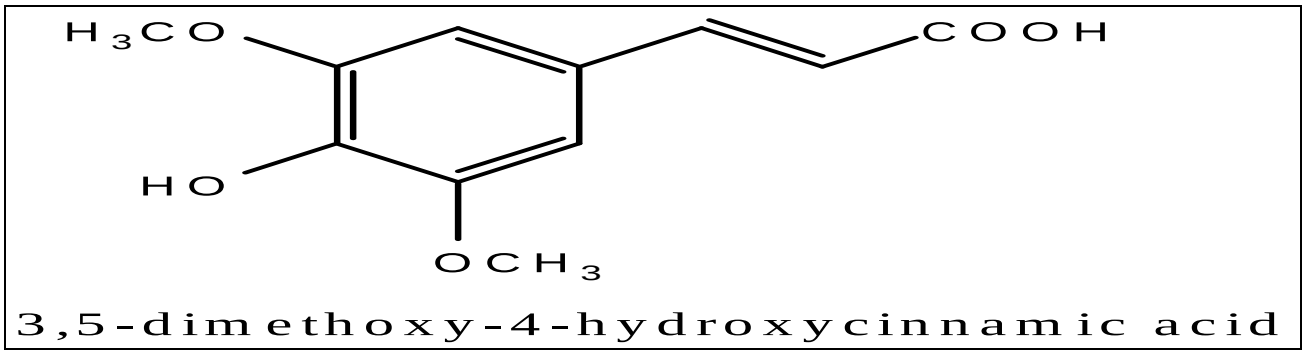


Fig. 1: Chemical structure of sinapic acid

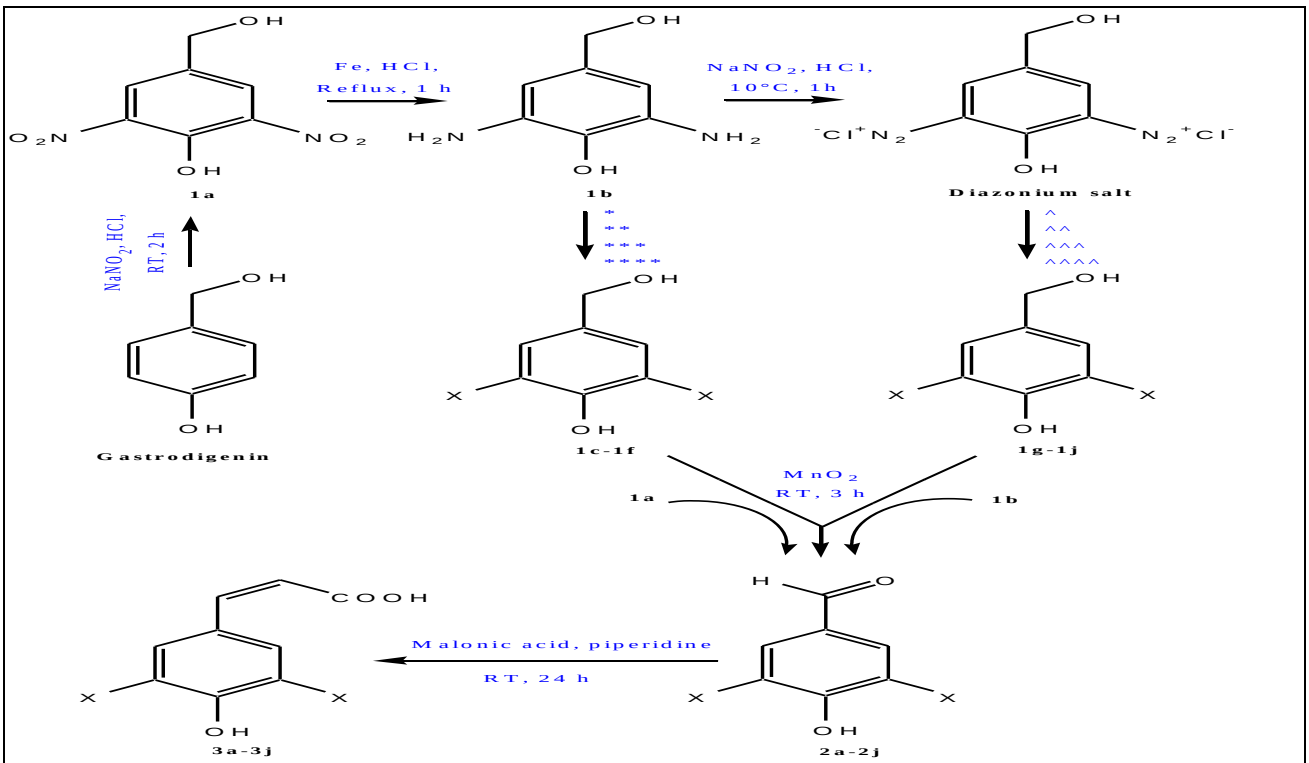
Many reports exploring in *vivo* and *in vitro* pharmacological properties of sinapic acid indicated that it has several effects including anti-inflammatory [8], antioxidant [9], antibacterial [10], analgesic [11], anxiolytic [12] and antidiabetic [13] effects. Until now, there is a limited data concerning the antitumor activity of sinapic acid. Based on literature, sinapic acid showed dose- and time-dependent cytotoxic effect against the following cancer cell lines: MDA MB 468, HBL 100 and T47D (breast), SW 480 and HT-29 (colon), HEp-2 (larynx), and HeLa (cervix) [14, 18].The aims of this work are to synthesize sinapic acid and nine of its novel analogues starting from gastrodigenin (4-hydroxybenzyl alcohol) through simple multi-step synthetic routes and to study their preliminary cytotoxic effect on four cancer cell lines, which are: HeLa (cervix), AMN3 (murine mammary adenocarcinoma), SKG (esophageal) and MCF-7 (breast) utilizing MTT viability assay.

Material and Methods

Chemicals employed in the synthesis of sinapic acid analogues were purchased from Sigma-Aldrich and Tokyo Chemical Industry while MTT stain (42000092-1) was supplied from Bio-World. Electrochemical CIA 9300 instrument was used to measure melting points of synthesized compounds and they were uncorrected. TLC tests were run on silica gel Si 60 F_{254} plates produced by Merck, and the mobile phase comprised of $CHCl_3$: acetone (4:1). IR spectra of the synthesized analogues were identified on Bruker-Alpha ATR while Varian UV/ Visible spectrophotometer were used to identify their UV spectra. ^{1}H-NMR (500 MHz, δ ppm) and ^{13}C-NMR (125 MHz, δ ppm) spectra were scanned on Bruker 500 MHz AVANCE III HD NMR Spectrometer using $CDCl_3$ as a solvent and TMS as an internal standard.. Shimadzu LCMS-2020 spectrometer was employed to record the mass spectra using electrospray ionization technique (ESI). These spectra are operated in positive mode $[M+H]^+$ and expressed as a mass-to-charge (m/z) ratio.

Synthesis

The synthetic pathway for the preparation of sinapic acid and its analogues is displayed in Scheme 1. IR spectral data of the intermediates (**1a-1j**), intermediates (**2a-2j**), and of the final products (**3a-3j**) are listed in Tables 1, 2 and 3, respectively. Physical properties of the prepared products are shown in Table 4.



1c, 2c, 3c: X = F; 1d, 2d, 3d: X = Br; 1e, 2e, 3e: X = Cl; 1f, 2f, 3f: X = I; 1g, 2g, 3g: X = OH; 1h, 2h, 3h: X = CN; 1i, 2i, 3i: X = OCH_3; 1j, 2j, 3j: X = OCH_2CH_3; 2a, 3a: X = NO_2; 2b, 3b: X = NH_2.

* Trifluoride diethyl etherate, tert-butyl nitrite, 5°C, 2 h; ** *p*-TsOH, *tert*-butyl nitrite, $NaBr$, $CuBr_2$, Grinding; *** *p*-TsOH, *tert*-butyl nitrite, benzyltriethylammonium chloride, $CuCl_2$, Grinding; **** $NaNO_2$, CH_2I_2, HOAc, RT, 1 h; ^ 2 h, 80°C; ^^ CuCN, RT, Overnight; ^^^ MeOH, 60°C, 24 h; ^^^^ EtOH, 60°C, 24 h.

Scheme 1: General synthetic pathway of sinapic acid and its analogues

Table 1: IR spectral data (v, str., cm^{-1}) of the intermediates (1a-1j)

Intermediate symbol	O-H (Phenol)	O-H (Alcohol)	C-H (Aromatic)	C-H (Alkyl)	C=C	Variable
2a	3323	3284	3021	2890	1580	1474, 1310 (NO_2)
2b	3317	3266	3026	2887	1567	3418, 3344 (N-H)
2c	3308	3270	3023	2872	1585	1230 (C-F)
2d	3321	3280	3018	2889	1581	1030 (C-Br)
2e	3330	3275	3027	2891	1571	1090 (C-Cl)
2f	3312	3269	3034	2888	1582	1003 (C-I)
2g	3304	3262	3021	2898	1578	----------
2h	3326	3274	3035	2889	1584	2228 (C≡N)
2i	3333	3272	3031	2891	1575	1312, 1048 (C-O-C)
2j	3320	3256	3019	2912	1583	1306,1037 (C-O-C)

Table 2: IR spectral data (v, str., cm^{-1}) of the intermediates (2a-2j).

Intermediate symbol	O-H (Phenol)	C-H (Aromatic)	C-H (Aldehyde)	C=O (Aldehyde)	C=C	Variable
2a	3283	3021	2720	1666	1558	1470, 1314 (NO_2)
2b	3298	3022	2712	1668	1556	3387, 3305 (N-H)
2c	3300	3020	2740	1676	1552	1229 (C-F)
2d	3301	3022	2734	1668	1557	1033 (C-Br)
2e	3300	3024	2737	1673	1550	1088 (C-Cl)
2f	3305	3028	2730	1668	1552	1012 (C-I)
2g	3278	3020	2724	1676	1546	----------
2h	3298	3027	2718	1670	1552	2234 (C≡N)
2i	3317	3031	2711	1665	1554	1310, 1040 (C-O-C)
2j	3313	3016	2717	1665	1550	1310,1031 (C-O-C)

Table 3: IR spectral data (v, str., cm^{-1}) of the final products (3a-3j).

Product symbol	O-H (Phenol)	O-H (Carboxylic)	C=O (Carboxylic)	C=C (Trans)	C=C (Aromatic)	Variable
3a	3345	2987	1701	1614	1533	1466, 1305 (NO_2)
3b	3316	2996	1700	1616	1527	3349 (N-H)
3c	3338	2978	1704	1612	1526	1233 (C-F)
3d	3333	2982	1700	1613	1514	1031 (C-Br)
3e	3340	2976	1709	1617	1520	1090 (C-Cl)
3f	3337	2988	1702	1614	1516	1010 (C-I)
3g	3304	2974	1689	1609	1522	----------
3h	3300	2992	1704	1612	1517	2229 (C≡N)
3i	3316	2977	1700	1620	1524	1318, 1035 (C-O-C)
3j	3328	2992	1706	1614	1515	1317,1030 (C-O-C)

Table 4: Physical properties of the intermediates (1a-1j and 2a-2j) and of the final products (3a-3j)

Compound symbol	Physical appearance	Yield %	mp °C	λmax (EtOH) nm	R_f
1a	White crystals	69	182-184	268	0.278
1b	White powder	52	206-208	256	0.216
1c	Off-white powder	67	137-139	316	0.326
1d	White crystals	52	64-67	240	0.329
1e	White crystals	46	98-100	242	0.305
1f	White crystals	81	134-136	235	0.372
1g	Off-white crystals	66	186-188	289	0.278
1h	White powder	46	103-105	240	0.356
1i	Gray powder	42	58-60	360	0.402
1j	Gray powder	54	74-76	376	0.436
2a	Pale yellow powder	87	105-107	520	0.286
2b	Off-white powder	80	134-136	321	0.189
2c	Off-white powder	88	119-121	289	0.347
2d	Pale yellow powder	79	178-180	488	0.356
2e	Off-white powder	85	155-158	279	0.343
2f	Light yellow powder	61	200-203	501	0.384
2g	Tan powder	72	114-116	347	0.278
2h	Tan crystals	69	178-182	306	0.359
2i	Colorless crystals	78	111-113	365	0.446
2j	Yellow powder	76	120-123	508	0.454
3a	Off-white powder	68	123-126	312	0.205
3b	White powder	62	153-156	287	0.114
3c	White powder	70	143-146	263	0.288
3d	Light yellow powder	68	196-198	423	0.294
3e	Off-white powder	71	169-172	279	0.343
3f	Off-white powder	52	215-218	312	0.303
3g	Tan powder	64	137-140	336	0.223
3h	Tan powder	58	190-193	367	0.288
3i	Light yellow powder	71	205-208	433	0.367
3j	Light yellow powder	72	189-193	427	0.434

Synthesis of 3, 5-dinitrogastrodigenin (1a)

To an aqueous solution of gastrodigenin (10 mmol, 1.24 g), a solution of $NaNO_2$ (25 mmol, 2.125 g) in 10 ml H_2O was added drop wise. The resultant solution was acidified with 1N HCl to achieve a pH value of 2 and then stirred for 2 h at room temperature (RT).The crude product was extracted by $CHCl_3$ (20 ml×2) and the organic layer was dried over anhydrous Na_2SO_4, followed by filtration and evaporation under vacuum. The product was recrystallized from MeOH [19].

*4-Hydroxymethyl-2,6-dinitrophenol (**1a**)*: 7.42 (s, 2H,Ar **H**), 6.12 (s, 1H, Ar-O**H**), 4.60 (s, 2H, Ar-C**H**$_2$), 2.24 (s, 1H,CH_2O**H**); 150.34 (Ar **C**-OH), 143.78 (Ar **C**-NO_2), 141.11 (Ar **C**-CH_2), 119.78 (Ar **C**), 67.95 (Ar C-**C**H_2); ESI: 215.05.

Synthesis of 3, 5-diaminogastrodigenin (1b)

A mixture of **1a** (10 mmol, 2.14 g) in 25 ml EtOH and iron powder (37.5 mmol, 2.1 g) was heated to 60°C using a water bath; to this, concentrated HCl (10 ml) was added dropwise over 30 min with stirring. The reaction mixture was refluxed for 1 h, poured into a cold H_2O (250 ml); the resultant mixture was neutralized with 1N NaOH and filtered. The crude product was extracted by ethyl acetate (25 ml×2) and the combined organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under vacuum. The final produce was recrystallized using a mixture of EtOH and H_2O [20].

*4-Hydroxymethyl-2,6-diaminophenol (**1b**)*: 7.36 (s, 2H, Ar **H**), 6.68 (s, 1H, Ar-O**H**), 4.58 (s, 2H, Ar-C**H**$_2$), 4.16 (s, 4H, Ar-N**H**$_2$), 2.28 (s, 1H,CH_2O**H**); 156.79 (Ar **C**-OH), 146.09 (Ar **C**-NH_2), 140.34 (Ar **C**-CH_2), 117.12 (Ar **C**), 68.48 (Ar C-**C**H_2); ESI: 155.25.

Synthesis of the diazonium Salt Solution

A solution of **1b** (10 mmol, 1.54 g) in a mixture of concentrated HCl (10 ml) and H_2O (8 ml) was placed in a salt-ice bath. As the solution temperature dropped to 0°C, a cold solution of $NaNO_2$ (24 mmol, 1.66 g) in 10 ml H_2O was added dropwise with stirring and the reaction temperature kept below 10°C. The resultant solution was stirred for 1 h at that temperature and then used immediately in the next step of the synthetic route [21].

Synthesis of 3, 5-difluorogastrodigenin (1c)

To a cold solution of **1b** (10 mmol, 1.54 g) in 50 ml CH_2Cl_2, boron trifluoride diethyl etherate (30 mmol, 5 ml) was added and the reaction temperature was kept below 5ºC. To this, *tert*-butyl nitrite (24 mmol, 2.2 ml) was slowly added from a graduated pipette with stirring in an ice bath. The reaction mixture was stirred for 2 h at that temperature and the formed solid was filtered, washed with H_2O and then with hexane. The crude product was re-dissolved in 60 ml CH_2Cl_2, refluxed for 24 h and filtered. The filtrate was evaporated under vacuum to afford the titled compound which was purified by recrystallization from a mixture of $CHCl_3$: ether (1:2) [22].

*4-Hydroxymethyl-2,6-difluorophenol (**1c**)*: 7.56 (s, 2H, Ar **H**), 6.66 (s, 1H, Ar-O**H**), 4.58 (s, 2H, Ar-C**H**$_2$), 2.34 (s, 1H,CH_2O**H**); 163.87 (Ar **C**-F), 154.01 (Ar **C**-OH), 140.36 (Ar **C**-CH_2), 117.69 (Ar **C**), 66.11 (Ar C-**C**H_2); ESI: 161.10.

Synthesis of 3, 5-dibromogastrodigenin (1d)

To an agate mortar, compound **1b** (10 mmol, 1.54 g), anhydrous *p*-TsOH (24 mmol, 4.12 g), *tert*-butyl nitrite (24 mmol, 2.2 ml), NaBr (24 mmol, 2.48 g), a catalytic amount of $CuBr_2$ and a few drops of H_2O were added and grinded. As the evolution of N_2 finished, H_2O (25 ml) was added and the crude product was extracted with CH_2Cl_2 (20 ml×2). The combined organic layer was dried over anhydrous $MgSO_4$, evaporated under vacuum and the titled product was recrystallized from $CHCl_3$ [23].

*4-Hydroxymethyl-2,6-dibromophenol (**1d**)*: 7.70 (s, 2H, Ar **H**), 6.43 (s, 1H, Ar-O**H**), 4.51 (s, 2H, Ar-C**H**$_2$), 2.46 (s, 1H,CH_2O**H**); 159.72 (Ar **C**-OH), 137.13 (Ar **C**-CH_2), 126.37 (Ar **C**-Br), 116.71 (Ar **C**), 60.95 (Ar C-**C**H_2); ESI: 283.05.

Synthesis of 3, 5-dichlorogastrodigenin (1e)

The same method used for the synthesis of **1d** was applied except that NaBr was replaced with benzyltriethylammonium chloride, $CuBr_2$ was replaced with $CuCl_2$, and the product recrystallized from a mixture of $CHCl_3$: EtOH (2:1).

*4-Hydroxymethyl-2,6-dichlorophenol **(1e)***: 7.24 (s, 2H, Ar **H**), 6.34 (s, 1H, Ar-O**H**), 4.47 (s, 2H, Ar-C**H**$_2$), 2.41 (s, 1H,CH$_2$O**H**); 153.38 (Ar **C**-OH), 133.58 (Ar **C**-CH$_2$), 120.44 (Ar **C**-Cl), 114.85 (Ar **C**), 60.41 (Ar C-**C**H$_2$); ESI: 194.05.

Synthesis of 3, 5-diiodogastrodigenin (1f)

A mixture of **1b** (10 mmol), $NaNO_2$ (50 mmol, 3.45 g), CH_2I_2 (40 mmol, 35.6 ml) in a solvent system consisted of CH_2Cl_2 (10 ml) and H_2O (10 ml) was stirred for 15 min. To this, HOAc (40 mmol, 2.52 ml) was slowly added in one portion, and then stirred for 1 h at RT. The resulted mixture was concentrated under vacuum and then hexane (20 ml) was added to the residue. The precipitate was filtered, washed with hexane and then with water. The product was recrystallized from a mixture of EtOH and ether [24].

*4-Hydroxymethyl-2,6-diiodophenol **(1f)***: 7.56 (s, 2H, Ar **H**), 6.39 (s, 1H, Ar-O**H**), 4.83 (s, 2H, Ar-C**H**$_2$), 2.42 (s, 1H,CH$_2$O**H**); 175.64 (Ar **C**-OH), 138.66 (Ar **C**-CH$_2$), 124.02 (Ar **C**), 87.46 (Ar **C**-I), 65.83 (Ar C-**C**H$_2$); ESI: 377.10.

Synthesis of 3, 5-dihydroxygastrodigenin (1g)

The temperature of the previously prepared diazonium salt solution was gradually raised to RT and then the solution was heated for 2 h at 80°C. The reaction mixture was left to cool and its pH was adjusted to 5 using 10% Na_2CO_3 solution. The crude yield was extracted with ethyl acetate (25 ml×2) and the combined organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under vacuum. The product was recrystallized from MeOH [25].

*5-Hydroxymethyl-benzene-1,2,3-triol **(1g)***: 7.14 (s, 2H, Ar **H**), 6.06 (s, 1H, Ar-O**H**), 5.38 (s, 2H, Ar-O**H**), 4.59 (s, 2H, Ar-C**H**$_2$), 2.15 (s, 1H,CH$_2$O**H**); 152.11, 133.79 (Ar **C**-OH), 144.17 (Ar **C**-CH$_2$), 110.75 (Ar **C**), 71.89 (Ar C-**C**H$_2$); ESI: 157.15.

Synthesis of Gastrodigenin3, 5-dicarbonitrile (1h)

To a freshly prepared aqueous solution of cuprous cyanide (20 mmol), cold diazonium salt solution (10 mmol) was added stepwise in an ice bath. The resulted solution was stirred at 20ºC for 40 min, at 70ºC for 40 min and overnight at RT. The solid was filtered under vacuum and the product was recrystallized from a mixture of EtOH and $CHCl_3$ (2:3) [26].

*2-Hydroxy-5-hydroxymethyl-isophthalonitrile **(1h)***: 7.63 (s, 2H, Ar **H**), 6.45 (s, 1H, Ar-O**H**), 4.88 (s, 2H, Ar-C**H**$_2$), 2.14 (s, 1H,CH$_2$O**H**); 166.08 (Ar **C**-OH), 148.22 (Ar **C**-CH$_2$), 123.68 (Ar-**C**N), 118.09 (Ar **C**), 101.93 (Ar **C**-CN), 72.44 (Ar C-**C**H$_2$); ESI: 175.05.

Synthesis of 3, 5-dimethoxygastrodigenin (1i)

To a cold solution of diazonium salt (10 mmol), absolute MeOH (50 ml) was added and then stirred for 24 h at 60ºC. The crude yield was extracted with ethyl acetate (20 ml×2), dried over anhydrous Na_2SO_4, and the solvent was evaporated under vacuum. The product was recrystallized from a mixture of $CHCl_3$ and ether (3:1) [27].

*4-Hydroxymethyl-2,6-dimethoxyphenol **(1i)***: 7.32 (s, 2H, Ar **H**), 6.40 (s, 1H, Ar-O**H**), 5.06 (s, 2H, Ar-C**H**$_2$), 3.95 (s, 6H, OC**H**$_3$), 2.26 (s, 1H,CH$_2$O**H**); 162.26 (Ar **C**-OH), 150.33 (Ar **C**-OCH$_3$), 131.60 (Ar **C**-CH$_2$), 122.26 (Ar **C**), 71.98 (Ar C-**C**H$_2$), 58.31 (Ar-O**C**H$_3$); ESI: 185.15.

Synthesis of 3, 5-diethoxygastrodigenin (1j)

The same method used for the synthesis of **1i** was applied except that absolute MeOH was replaced with absolute EtOH and the product was recrystallized from ether.

*4-Hydroxymethyl-2,6-diethoxyphenol **(1j)***: 7.08 (s, 2H, Ar **H**), 6.24 (s, 1H, Ar-O**H**), 5.12 (s, 2H, Ar-C**H**$_2$), 4.30 (m, 4H, *J*= 6.8 Hz, OC**H**$_2$), 2.26 (s, 1H,CH$_2$O**H**), 1.56 (t, 6H, , *J*= 6.8 Hz, C**H**$_3$); 160.15 (Ar **C**-OH), 149.89 (Ar **C**-OCH$_2$), 131.79 (Ar **C**-CH$_2$), 109.71 (Ar **C**), 71.84 (Ar C-**C**H$_2$), 63.85 (Ar-O**C**H$_2$), 18.37 (**C**H$_3$); ESI: 213.15.

General Method for Synthesis of Syringaldehyde and its Analogues (2a-2j)

A suspension was prepared from 10 mmol of each of intermediates **1a-1j** and MnO_2 (4.35 g, 50 mmol) in 50 ml $CHCl_3$. The suspension was stirred for 3 h at RT and then filtered through a plug of purified glass wool and later washed with warm $CHCl_3$ (2×10 ml). The filtrate was concentrated to dryness under vacuum, re-dissolved in 30 ml acetone and filtered. The solvent was evaporated and

the final product was then recrystallized from EtOH [28].

4-Hydroxy-3, 5-dinitrobenzaldehyde ***(2a)***: 10.14 (s, 1H, Ar-C**H**O), 8.92 (s, 2H, Ar **H**), 6.08 (s, 1H, Ar-O**H**); 192.69 (Ar-**C**HO), 151.78 (Ar **C**-OH), 142.82 (Ar **C**-NO_2), 133.03 (Ar **C**-CHO), 125.73 (Ar **C**); ESI: 213.20.

4-Hydroxy-3,5-diaminobenzaldehyde ***(2b)***: 9.92 (s, 1H, Ar-C**H**O), 7.58 (s, 2H, Ar **H**), 6.68 (s, 1H, Ar-O**H**), 4.12 (s, 4H, Ar-N$\mathbf{H}_2$); 190.15 (Ar-**C**HO), 164.38 (Ar **C**-OH), 145.75 (Ar **C**-NH_2), 131.84 (Ar **C**-CHO), 120.27 (Ar **C**); ESI: 153.15.

4-Hydroxy-3, 5-difluorobenzaldehyde ***(2c)***: 10.04 (s, 1H, Ar-C**H**O), 7.78 (s, 2H, Ar **H**), 6.60 (s, 1H, Ar-O**H**); 187.44 (Ar-**C**HO), 157.74 (Ar **C**-F), 150.24 (Ar **C**-OH), 137.54 (Ar **C**-CHO), 122.05 (Ar **C**); ESI: 159.05.

4-Hydroxy-3, 5-dibromobenzaldehyde ***(2d)***: 9.82 (s, 1H, Ar-C**H**O), 7.74 (s, 2H, Ar **H**), 6.41 (s, 1H, Ar-O**H**); 192.26 (Ar-**C**HO), 164.62 (Ar **C**-OH), 136.85 (Ar **C**-CHO), 126.68 (Ar **C**-Br), 118.56 (Ar **C**); ESI: 281.05.

4-Hydroxy-3, 5-dichlorobenzaldehyde ***(2e)***: 10.15 (s, 1H, Ar-C**H**O), 7.77 (s, 2H, Ar **H**), 6.30 (s, 1H, Ar-O**H**); 196.67 (Ar-**C**HO), 159.61 (Ar **C**-OH), 132.83 (Ar **C**-CHO), 120.76 (Ar **C**-Cl), 117.89 (Ar **C**); ESI: 192.25.

4-Hydroxy-3, 5-diiodobenzaldehyde ***(2f)***: 9.95 (s, 1H, Ar-C**H**O), 7.78 (s, 2H, Ar **H**), 6.37 (s, 1H, Ar-O**H**); 190.79 (Ar-**C**HO), 182.05 (Ar **C**-OH), 133.43 (Ar **C**-CHO), 126.82 (Ar **C**), 88.86 (Ar **C**-I); ESI: 375.25.

3, 4, 5-Trihydroxybenzaldehyde ***(2g)***: 10.18 (s, 1H, Ar-C**H**O), 7.54 (s, 2H, Ar **H**), 6.38 (s, 1H, Ar-O**H**), 5.62 (s, 2H, Ar-O**H**); 198.07 (Ar-**C**HO), 155.12, 140.25 (Ar **C**-OH), 137.70 (Ar **C**-CHO), 115.50 (Ar **C**); ESI: 155.10.

5-Formyl-2-hydroxy-isophthalonitrile ***(2h)***: 10.12 (s, 1H, Ar-C**H**O), 7.98 (s, 2H, Ar **H**), 6.22 (s, 1H, Ar-O**H**); 192.78 (Ar-**C**HO), 176.66 (Ar **C**-OH), 140.80 (Ar **C**-CHO), 123.83 (Ar-**C**N), 120.90 (Ar **C**), 100.76 (Ar **C**-CN); ESI: 173.20.

4-Hydroxy-3,5-dimethoxybenzaldehyde ***(2i)***: 9.92 (s, 1H, Ar-C**H**O), 7.78 (s, 2H, Ar **H**), 6.38 (s, 1H, Ar-O**H**), 3.98 (s, 6H, OC$\mathbf{H}_3$); 190.11 (Ar-**C**HO), 157.81 (Ar **C**-OH), 149.77 (Ar **C**-OCH_3), 134.35 (Ar **C**-CHO), 126 (Ar **C**), 58.47 (Ar-O**C**H_3); ESI: 183.05.

4-Hydroxy-3,5-diethoxybenzaldehyde ***(2j)***: 9.88 (s, 1H, Ar-C**H**O), 7.80 (s, 2H, Ar **H**), 6.22 (s, 1H, Ar-O**H**), 4.25 (m, 4H, *J*= 6.8 Hz,OC$\mathbf{H}_2$),1.60 (t, 6H, *J*= 6.8 Hz, C$\mathbf{H}_3$); 189.68 (Ar-**C**HO), 157.91 (Ar **C**-OH), 144.73 (Ar **C**-OCH_2), 127.23 (Ar **C**-CHO), 112.69 (Ar **C**), 65.17 (Ar-O**C**H_2), 16.46 (**C**H_3); ESI: 211.15.

General Method for Synthesis of sinapic Acid and its Analogues (3a-3j)

To a solution prepared from 10 mmol of each of intermediates **2a**-**2j** in 10 ml pyridine, mixture of malonic acid (20 mmol, 2.08 g) and piperidine (2.2 mmol, 0.2 ml) in 10 ml pyridine was added. The reaction mixture was stirred for 24 h at RT and to this, concentrated HCl (6.6 ml) and then H_2O (100 ml) were added sequentially. The crude product was filtered and then recrystallized from hot $CHCl_3$ in a product-solvent ratio ranging from 1:20 to 1:50 [8].

3,5-Dinitro-4-hydroxycinnamic acid ***(3a)***: 12.69 (s, 1H, COO**H**), 8.48 (s, 2H, Ar **H**), 7.58 (d, 1H, *J*= 16.6 Hz, Ar-C**H**=CH), 6.62 (d, 1H, *J*= 16.6 Hz, Ar-CH=C**H**), 6.12 (s, 1H, Ar-O**H**); 174.54 (**C**OOH), 147.96 (Ar **C**-OH), 140.07 (Ar C-**C**H=), 138.15 (Ar**C**-CH=), 133.23 (Ar **C**-NO_2), 120.12 (Ar **C**), 107.78 (Ar-CH=**C**H); ESI: 255.30.

3,5-Diamino-4-hydroxycinnamic acid ***(3b)***: 12.36 (s, 1H, COO**H**), 7.72 (d, 1H, *J*= 16.2 Hz, Ar-C**H**=CH), 7.08 (s, 2H, Ar **H**), 6.54 (d, 1H, *J*= 16.2 Hz, Ar-CH=C**H**), 6.16 (s, 1H, Ar-O**H**), 4.00 (s, 4H, Ar-N$\mathbf{H}_2$); 170.12 (**C**OOH), 150.58 (Ar **C**-OH), 140.36 (Ar **C**-NH_2), 136.89 (Ar C-**C**H=), 130.21 (Ar**C**-CH=), 123.46 (Ar **C**), 100.67 (Ar-CH=**C**H); ESI: 195.15.

3,5-Difluoro-4-hydroxycinnamic acid ***(3c)***: 12.38 (s, 1H, COO**H**), 7.74 (d, 1H, *J*= 16.4 Hz, Ar-C**H**=CH), 7.16 (s, 2H, Ar **H**), 6.68 (d, 1H, *J*= 16.2 Hz, Ar-CH=C**H**), 6.23 (s, 1H, Ar-O**H**); 171.65 (**C**OOH), 155.05 (Ar **C**-F), 142.88 (Ar **C**-OH), 139.92 (Ar C-**C**H=), 126.33 (Ar**C**-CH=), 119.91 (Ar **C**) , 109.11 (Ar-CH=**C**H); ESI: 201.10.

3,5-Dibromo-4-hydroxycinnamic acid ***(3d)***: 12.32 (s, 1H, COO**H**), 7.70 (d, 1H, *J*= 16.9 Hz, Ar-C**H**=CH), 7.18 (s, 2H, Ar **H**), 6.59 (d, 1H, *J*= 16.9 Hz, Ar-CH=C**H**), 6.38 (s, 1H, Ar-O**H**); 175.78 (**C**OOH), 165.98 (Ar **C**-OH), 150.17 (Ar C-**C**H=), 129.24 (Ar **C**-Br), 125.55 (Ar **C**-CH=), 120.16 (Ar **C**), 110.07 (Ar-CH=**C**H); ESI: 323.10.

*3,5-Dichloro-4-hydroxycinnamic acid (**3e**)*: 12.35 (s, 1H, COO**H**), 7.71 (d, 1H, *J*= 16.1 Hz, Ar-C**H**=CH),7.23 (s, 2H, Ar **H**), 6.62 (d, 1H, *J*= 16.1 Hz, Ar-CH=C**H**), 6.29 (s, 1H, Ar-O**H**); 170.23 (**C**OOH), 162.01 (Ar **C**-OH), 152.78 (Ar C-**C**H=),135.06 (Ar**C**-CH=), 125.87 (Ar **C**-Cl), 118.28 (Ar **C**), 108.44 (Ar-CH=**C**H); ESI: 234.25.

*3,5-Diiodo-4-hydroxycinnamic acid (**3f**)*: 12.38 (s, 1H, COO**H**), 7.72 (d, 1H, *J*= 16.4 Hz, Ar-C**H**=CH), 7.27 (s, 2H, Ar **H**), 6.56 (d, 1H, *J*= 16.4 Hz, Ar-CH=C**H**), 6.38 (s, 1H, Ar-O**H**); 175.45 (Ar **C**-OH), 170.44 (**C**OOH), 153.98 (Ar C-**C**H=), 131.87 (Ar **C**-CH=), 123.96 (Ar **C**), 110.61 (Ar-CH=**C**H), 88.16 (Ar **C**-I); ESI: 417.05.

*3,4,5-Trihydroxycinnamic acid (**3g**)*: 12.09 (s, 1H, COO**H**), 7.70 (d, 1H, *J*= 16.2 Hz, Ar-C**H**=CH), 7.23 (s, 2H, Ar **H**), 6.55 (d, 1H, *J*= 16.2 Hz, Ar-CH=C**H**), 6.32 (s, 1H, Ar-O**H**), 5.61 (s, 2H, Ar-O**H**); 168.22 (**C**OOH), 158.67, 146.78 (Ar **C**-OH), 150.03 (Ar C-**C**H=), 133.36 (Ar **C**-CH=), 127.22 (Ar **C**), 111.91 (Ar-CH=**C**H); ESI: 197.10.

*3,5-Dicyano-4-hydroxycinnamic acid (**3h**)*: 12.63 (s, 1H, COO**H**), 7.83 (s, 2H, Ar **H**), 7.72 (d, 1H, *J*= 16.8 Hz, Ar-C**H**=CH), 6.65 (d, 1H, *J*= 16.8 Hz, Ar-CH=C**H**), 6.24 (s, 1H, Ar-O**H**); 172.45 (**C**OOH), 166.83 (Ar **C**-OH), 149.69 (Ar C-**C**H=), 140.24 (Ar **C**), 131.21 (Ar **C**-CH=), 122.65 (Ar-CH=**C**H), 118.11 (Ar-**C**N), 102.34 (Ar **C**-CN); ESI: 215.05.

*3,5-Dimethoxy-4-hydroxycinnamic acid (**Sinapic acid**, **3i**)*: 12.46 (s, 1H, COO**H**), 7.69 (d, 1H, *J*= 16.4 Hz, Ar-C**H**=CH), 7.21 (s, 2H, Ar **H**), 6.66 (d, 1H, *J*= 16.4 Hz, Ar-CH=C**H**), 6.38 (s, 1H, Ar-O**H**), 3.94 (s, 6H, OC**H**$_3$); 170.23 (**C**OOH), 162.41 (Ar **C**-OH), 152.78 (Ar **C**-OCH$_3$), 147.07 (Ar C-**C**H=), 135.56 (Ar **C**-CH=), 120.89 (Ar **C**), 114.81 (Ar-CH=**C**H), 58.21 (Ar-O**C**H$_3$); ESI: 225.05.

*3,5-Diethoxy-4-hydroxycinnamic acid (**3j**)*: 12.46 (s, 1H, COO**H**), 7.74 (d, 1H, *J*= 16.3 Hz, Ar-C**H**=CH), 7.23 (s, 2H, Ar **H**), 6.74 (d, 1H, *J*= 16.3 Hz, Ar-CH=C**H**), 6.20 (s, 1H, Ar-O**H**), 4.26 (m, 4H, *J*= 7.0 Hz, OC**H**$_2$), 1.43 (t, 6H, *J*= 7.0 Hz, C**H**$_3$); 170.34 (**C**OOH), 154.67 (Ar **C**-OH), 148.98 (Ar **C**-OCH$_2$), 142.27 (Ar C-**C**H=), 137.83 (Ar **C**-CH=), 109.01 (Ar **C**), 69.18 (Ar-O**C**H$_2$), 16.12 (**C**H$_3$); ESI: 253.15.

Preliminary Cytotoxicity Study

Cytotoxicity study was conducted based on the MTT method reported by Mustafa *et al.* (2018) to test the ability of the synthesized compounds to induce the cytotoxicity utilizing 5-fluorouracil and DMSO as positive and negative controls respectively [29]. This test was applied on the following cancer cell lines: HeLa (cervix), AMN3 (murine mammary adenocarcinoma), SKG (esophageal) and MCF-7 (breast). MTT test was applied using 96-well plates and a specific cell line was seeded at 1×104 cells/well. Cell line was treated after one day separately with each of the sinapic acid and its synthesized analogues. The cell viability was tested after 3 days by taking off the medium, adding 28 µl of MTT solution (2 mg/ml) and then the cells were incubated at 37°C for 1.5 hours.

Solid remaining in the wells, as the MTT solution was removed, was solubilized by DMSO (130 µl) followed by incubation for 15 minutes at 37°C. Using a microplate reader adjusted at 492 nm, the absorbance was detected and the assay was performed in triplicate. The inhibition rate of cell growth was calculated according to the following equation: Inhibition rate = (A − B)/A × 100, where A is the average optical density of untreated wells, and B is the average optical density of treated wells [29].

Results and Discussion

Synthesis

Synthesis of sinapic acid and its analogues starting from gastrodigenin was carried out through 6 successive synthetic steps as shown in Scheme 1. The nitration of gastrodigenin afforded compound **1a**, which was subject to Bechamp reduction reaction to form compound **1b**. The amino groups of **1b** were diazonated, and the resultant diazonium salt compound was transformed via Sandmeyer reaction to give compounds **1c**-**1h**.

Aryl ethers containing compounds **1i** and **1j** were prepared via nucleophilic aromatic substitution reaction of the diazonium salt with MeOH and EtOH, respectively. The selective oxidation of primary hydroxyl group found in compounds **1a**-**1j** using MnO_2 afforded compounds **2a**-**2j**, which were coupled with malonic acid in the presence of organic base to form compounds **3a**-**3j** in a good yield. Compounds **1a**-**1e**, **1h**, **1j**, **2b**, **2h**, **2j**, **3a-3h,** and **3j** are novel. Compounds **1f**, **1g**, **1i**, **2c**, and **2e-2g** are available

commercially, but there are no methods in the literature for their preparation. There are methods for the preparation of compounds **2a**, **2d**, and **2i**, but those used in this work are different. Finally, only compound **3i** has an established synthetic method which was utilized in this work [8]. The chemical structures of the final products (**3a**-**3j**) are displayed in Figure 2.

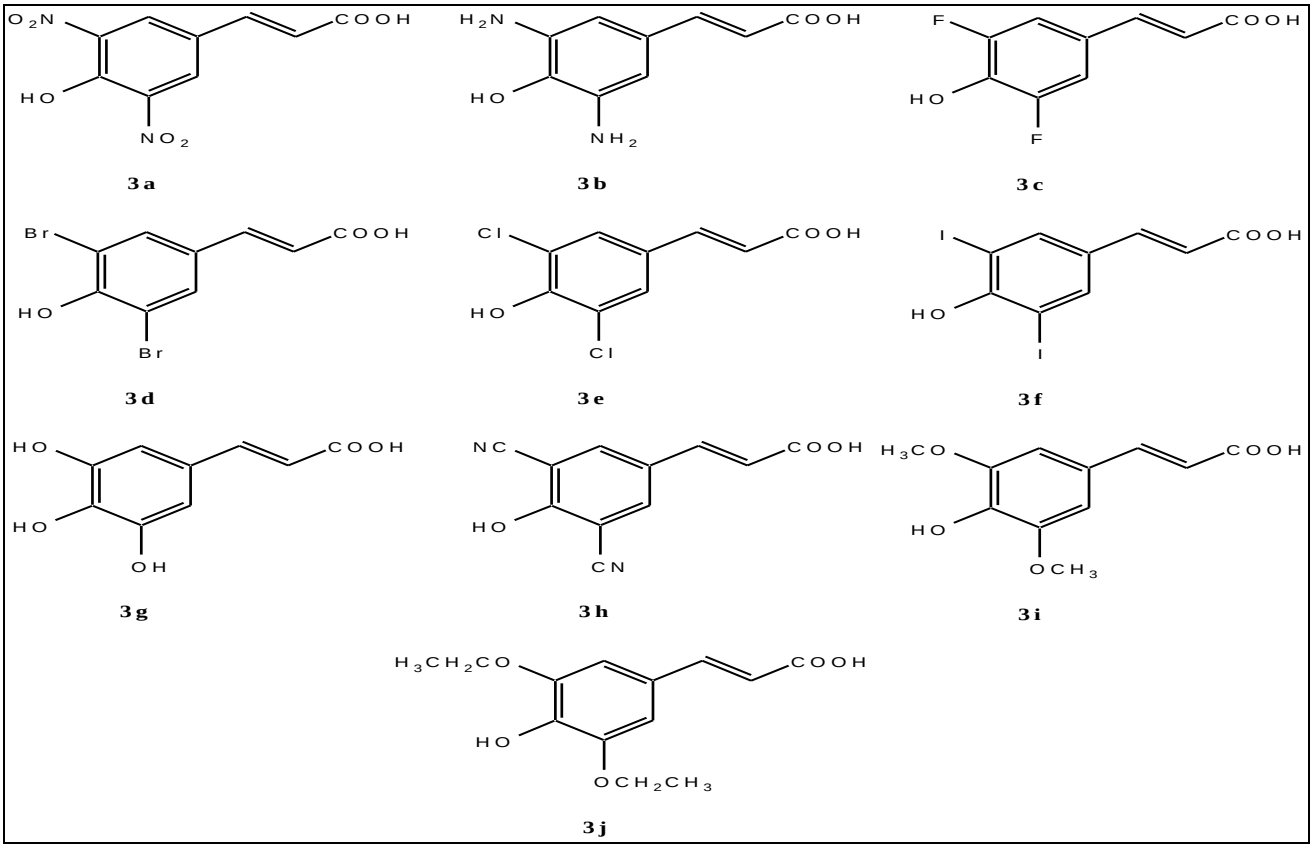


Fig. 2: Chemical structures of the final products (3a-3j)

In vitro Cytotoxicity Study

Sinapic acid and its synthesized analogues were scanned for their preliminary cytotoxic activity utilizing MTT test against four cancer cell lines, which are: HeLa (cervix), AMN3 (murine mammary adenocarcinoma), SKG (esophageal) and MCF-7 (breast).Eight serial concentrations (3.125, 6.25, 12.5, 25, 50, 100, 200, 400 µg/ml) of tested compounds, DMSO as a negative control and 5-fluorouracil as a positive control were applied in this assay. The results presented in Table 5 indicate that sinapic acid analogues **3a**, **3c**, **3d**, **3e** and **3h** have IC_{50} values lower than that of 5-fluorouracil against the tested cancer cell lines. Also, the results revealed that sinapic acid itself and the remaining synthesized analogues have IC_{50} values higher than that of 5-fluorouracil. Accordingly, compounds **3a**, **3c**, **3d**, **3e** and **3h** can be recommended as potential cytotoxic agents. To investigate the role of different substituent on cytotoxic activity of the synthesized compounds, data in Table 5 was analysed. This study proposes that there is an inverse relationship between the nucleophilicity of the oxygen of phenolic hydroxyl group and the preliminary cytotoxic activity of the synthesized products. The electron withdrawing substituent's NO_2, F, Br, Cl and CN in ortho positions to phenolic hydroxyl group in compounds **3a**, **3c**, **3d**, **3e** and **3h** respectively**,** reduced the nucleophilicity of phenolic oxygen and the cytotoxic activity against the tested cell lines was enhanced. The electron donating substituents NH_2, OH, OCH_3 and OCH_2CH_3 in ortho positions to phenolic hydroxyl group in compounds **3b**, **3g**, **3i** and **3j** respectively**,** increased the nucleophilicity of phenolic oxygen and the cytotoxic activity against the tested cell lines was lower than that with electron withdrawing groups. The exception to this assumption is the iodide substituent in compound **3f**; although it is an electron withdrawing group, the cytotoxic activity of compound **3f** was lower than that obtained with the other electron withdrawing substituents. This may be attributed to the low electro negativity of this substituent which results in compromised effect on the nucleophilicity of phenolic oxygen.

Table 5: Mean ± SD IC_{50} values of 5- Fluorouracil as a positive control, sinapic acid and its analogues against HeLa, AMN3, SKG and MCF-7 cancer cell lines for triplicate trials.

Compound symbol	MCF-7 IC_{50}(µg/ml)	AMN3 IC_{50} (µg/ml)	SKG IC_{50} (µg/ml)	HeLa IC_{50} (µg/ml)
Positive control	14.65 ± 1.033	15.47 ± 1.042	8.74 ± 1.213	12.68 ± 0.957
3a	8.97 ± 2.649	14.56 ± 1.455	7.91 ± 2.115	10.34 ± 1.243
3b	177.34 ± 7.001	212.34 ± 3.258	237.74 ± 1.418	112.57 ± 4.111
3c	14.22 ± 2.687	13.33 ± 4.437	8.03 ± 5.114	12.13 ± 2.030
3d	14.63 ± 4.571	12.15 ± 2.904	8.54 ± 3.737	11.22 ± 2.451
3e	8.32 ± 1.889	13.59 ± 2.005	8.53 ± 1.213	9.78 ± 1.236
3f	56.56 ± 2.681	79.88 ± 3.983	98.55 ± 6.422	106.02 ± 1.889
3g	146.12 ± 5.349	204.21 ± 4.016	256.66 ± 3.778	211.09 ± 2.566
3h	13.47 ± 1.983	12.01 ± 2.544	8.12 ± 0.233	11.86 ± 2.455
3i	109.54 ± 3.119	192.94 ± 2.661	178.65 ± 4.783	158.78 ± 3.275
3j	167.76 ± 2.565	190.16 ± 5.603	226.34 ± 2.019	210.04 ± 1.882

Conclusion

The synthetic steps utilised in this work were easy to apply and successful in yielding the required products with sufficient purity as indicated by their analysis spectra. The simple synthesis did not prevent these compounds from having good cytotoxic activity as shown by the results of the MTT assay. These results revealed that sinapic acid analogue **3a**, **3c**, **3d**; **3e** and **3h** have IC_{50} values lower than that of positive control on the tested cancer cell lines. Therefore, these compounds can be used as a starting point for cytotoxic agent development.

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