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C-Glycosylated cinnamoylfuran derivatives as novel anti-cancer agents†

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A series of C-glycosylated cinnamoylfuran derivatives were synthesized in excellent yield from free sugars. The C-glycosylated furan derivatives were prepared under aqueous reaction conditions. The anticancer potentials of the synthesized compounds were evaluated on the basis of their comparative cytotoxic effects on cancer cells (MCF-7 and HeLa) and normal cells (NKE). Among 28 compounds evaluated for their cytotoxic effects on cancer cells, three compounds (compounds **8**, **24** and **28**) were shown to have significant cytotoxic effects on MCF-7 and HeLa cell lines and comparatively less toxicity against normal NKE cell line. Based on its selectivity index, compound **24** was considered the most promising anticancer agent amongst the above three compounds. Further biochemical studies with compound **24** showed that both intrinsic and extrinsic pathways of apoptosis contribute to compound **24** mediated cytotoxicity.

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Introduction

Cancer is a leading cause of death worldwide, representing a number of diseases due to uncontrolled cell division.¹ Cervical cancer and breast cancer are the most frequent among several organ-specific cancers found in women throughout the world and their management strategies are quite expensive.^{2,3} Although several anticancer therapeutics are available in clinics, they suffer from poor prognosis due to their nonspecific action and cytotoxic effects towards normal cells.^{4–6} The use of many commonly used chemotherapeutic agents is limited because of drug resistance.⁷ Therefore, the development of novel therapeutics with better efficacy and lower toxicity is a thrusting area in medicinal chemistry. Over the years, several chemotherapeutic agents have been developed against a number of organ-specific cancers, taking their lead from natural products or synthetic intermediates.^{8,9} In the recent past, C-glycosylated heterocycles, such as C-glycosylfuran derivatives and related compounds, have been reported for their therapeutic potential.^{10–15} In addition, a variety of cinnamoylated C-glycoside derivatives have been synthesized with a similar sugar moiety but different aryl groups and evaluated for their potential as anti-mycobacterial agents,^{16,17} anti-cancer agents,¹⁸ lectin inhibitors,^{19,20} enzyme inhibitors,²¹ and anti-

filial agents.²² Inspired by these earlier reports, it was decided to synthesize a series of C-glycosylated cinnamoylfuran derivatives to evaluate their potential against cancer cell lines to develop novel anticancer agents. It was envisioned that the presence of a C-glycosylated furan moiety as well as an aryl cinnamoyl functionality in the molecules could improve their efficacy to act as effective cytotoxic agents against cancer cells. The C-glycosylated furan derivatives containing an α -methyl-carbonyl functionality were prepared from commercially available reducing sugars under aqueous reaction conditions, following an earlier report.^{23,24} The furan derivatives were treated with a number of aryl aldehydes in the presence of a base to furnish the desired compounds. Some selected C-cinnamoylated products were acetylated to check whether the O-acetyl group has any influence on the biological activities. The synthesized compounds (**7–34**) were evaluated for their cytotoxicity potential against two cancer cell lines (MCF-7 and HeLa) as well as a normal cell line (NKE). The synthesis of a series of novel C-glycosyl cinnamoylfuran derivatives and their potential as anticancer agents are reported herein.

Results and discussion

Chemistry

Synthesis of C-glycosylated cinnamoylfuran derivatives. A series of C-glycosylated cinnamoyl furan derivatives (**7–34**) were synthesized from D-glucose, D-galactose and D-xylose following a two-step reaction sequence. Following earlier reported reaction conditions,²³ treatment of D-glucose (**1**), D-galactose (**2**) and D-xylose (**3**) with pentane-2,4-dione in the presence of CeCl₃·7H₂O in water furnished the corresponding C-glycosylated furan derivatives, such as 3-acetyl-5-C-glycosyl-2-methylfurans **4** (90%)

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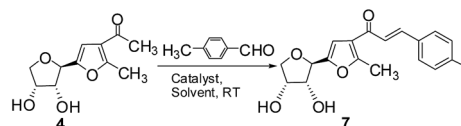
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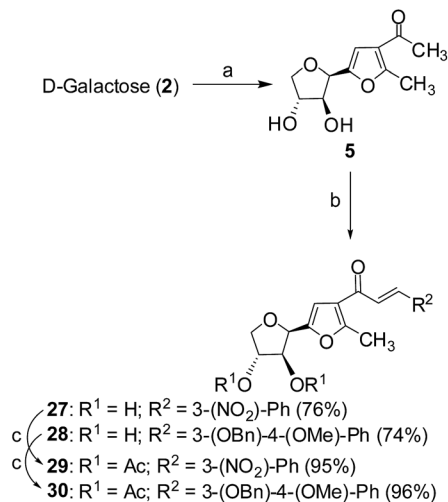


Table 1 Optimization of the preparation of *C*-glycosylated cinnamoyl furan derivatives

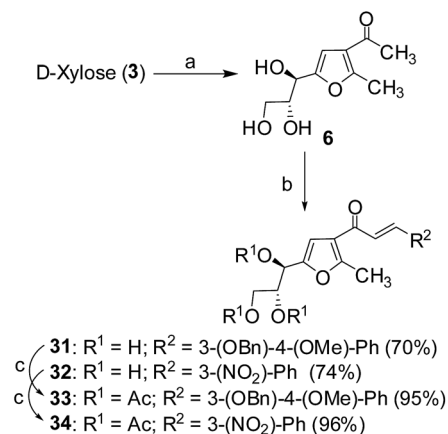


Sl. no.	Base	Equiv.	Solvent	Time (h)	Yield (%)
1	NaOH	1.0	CH ₃ OH	12	50
2	NaOCH ₃	1.0	CH ₃ OH	12	75
3	NaOC ₂ H ₅	1.0	C ₂ H ₅ OH	16	60
4	Piperidine	1.0	CH ₃ OH	24	50
5	DBU	1.0	CH ₃ OH	24	40

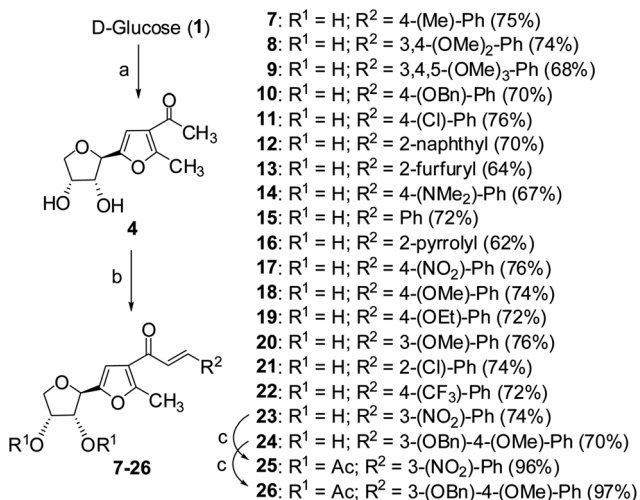
and **5** (90%) and 3-acetyl-5-(1,2,3-trihydroxypropyl)-2-methylfuran **6** (85%), respectively, with an α -methylcarbonyl functionality attached to them. In order to synthesize a series of cinnamoyl derivatives under a base mediated aldol condensation of aryl aldehydes with compounds **4–6**, compound **4** was initially allowed to react with 4-methylbenzaldehyde in the presence of a series of bases (NaOH, NaOCH₃, NaOEt, piperidine, DBU) (1.0 equiv.) in alcohol (Table 1). After a set of optimizations, it was found that the use of 1.0 equiv. of sodium methoxide furnished an excellent yield (75%) of the desired product (**7**) in 12 h. Therefore, compound **4** was allowed to condense with a series of aromatic aldehydes in the presence of sodium methoxide in methanol to give *C*-glycosylated cinnamoyl furan derivatives (**7–24**) in excellent yield. The formation of the *trans*-cinnamoyl double bonds (*E*-isomers) in the molecules was confirmed from a ¹H NMR spectral analysis [doublets with large coupling constant ($J = 15.5–16.0$ Hz)]. It is noteworthy that the formation of other isomers was not observed under the aldol reaction conditions. Acetylation of compounds **23** and **24** using acetic



Scheme 2 Reagents and conditions: (a) pentane-2,4-dione, CeCl₃·7H₂O, H₂O, 90 °C, 6 h, 90%; (b) ArCHO, CH₃ONa, CH₃OH, rt, 12 h; (c) acetic anhydride, pyridine, rt, 3 h.



Scheme 3 Reagents and conditions: (a) pentane-2,4-dione, CeCl₃·7H₂O, H₂O, 90 °C, 6 h, 85%; (b) ArCHO, CH₃ONa, CH₃OH, rt, 12 h; (c) acetic anhydride, pyridine, rt, 3 h.



Scheme 1 Reagents and conditions: (a) pentane-2,4-dione, CeCl₃·7H₂O, H₂O, 90 °C, 6 h, 90%; (b) ArCHO, CH₃ONa, CH₃OH, rt, 12 h; (c) acetic anhydride, pyridine, rt, 3 h.

anhydride and pyridine furnished acetylated derivatives **25** and **26** in quantitative yield (Scheme 1). Following similar reaction conditions, compounds **27** and **28** were prepared from compound **5** in very good yield, which were then acetylated to give compounds **29** and **30** (Scheme 2). Compounds **31** and **32** were synthesized from *D*-xylose derived compound **6** in very good yield, which were acetylated to give compounds **33** and **34** (Scheme 3). Earlier it was observed that *O*-acetylated cinnamoyl *C*-glycoside derivatives showed better antifilarial activities *via* an apoptotic pathway than non-*O*-acetylated compounds.²² This could happen due to the increase in lipophilicity in the compounds because of the presence of acetyl groups. Based on this observation, we have prepared some acetylated derivatives (**25**, **26**, **29**, **30**, **33** and **34**) to compare the cytotoxic activities of *O*-acetylated and non-*O*-acetylated compounds against cancer and normal cell lines. All synthesized compounds were characterized using spectral analysis.



Table 2 The IC₅₀ (μM) values of the compounds for the inhibition of proliferation^a

Compd	MCF-7	HeLa	NKE
7	≥60	≥60	—
8	17.77 ± 1.46	15.98 ± 1.85	46.03 ± 10.56
9	≥60	10.93 ± 5.36	109 ± 7.85
10	64.54 ± 13.16	≥60	75.07 ± 27.83
11	≥60	≥60	—
12	≥60	≥60	—
13	≥60	≥60	—
14	68.75 ± 12.84	81.48 ± 76.22	≥60
15	90.77 ± 105.53	35.93 ± 10.13	59.05 ± 59.05
16	54.07 ± 6.49	≥60	≥60
17	≥60	≥60	—
18	≥60	≥60	—
19	43.49 ± 7.39	47.58 ± 10.37	≥60
20	≥60	≥60	—
21	45.37 ± 14.31	≥60	99.92 ± 27.89
22	33.0 ± 1.5	≥60	24.38 ± 11.38
23	≥60	≥60	—
24	9.588 ± 5.25	14.22 ± 3.94	54.14 ± 1.56
25	≥60	≥60	—
26	≥60	≥60	—
27	32.35 ± 3.6	≥60	≥60
28	22.98 ± 4.19	22.29 ± 4.03	40.29 ± 11.87
29	34.79 ± 6.86	≥60	≥60
30	≥60	≥60	—
31	43.84 ± 7.66	30.25 ± 6.76	30.23 ± 23
32	≥60	≥60	—
33	91.27 ± 53.63	≥60	23.84 ± 17.45
34	≥60	≥60	—
Etoposide	24.77 ± 3.4	29.12 ± 8.56	—

^a IC₅₀ = concentration at which 50% inhibition in motility was achieved.

Biology

Cytotoxic effect of C-glycosyl cinnamoylfuran derivatives by MTT cell proliferation assay. The newly synthesized C-glycosyl-3-cinnamoylfuran derivatives (7–34) were screened *in vitro* for their anti-proliferative effect on MCF-7 (Human breast carcinoma), HeLa (Human cervical carcinoma) and NKE (Normal Kidney Epithelial) cells. MCF-7 is the most studied human breast cancer cell line and results from this cell line have had a fundamental impact upon breast cancer research and patient outcomes. The MCF-7 cell line is one of a very few breast cancer cell lines that expresses substantial levels of ER, mimicking the majority of invasive human breast cancers that express ER.²⁵ Similarly, HeLa has been widely used as a model for cervical cancer over the past 50 years of cervical cancer research.²⁶ Owing to these facts we chose these two cells lines for our experiments. Etoposide was used as a standard control, considering its tremendous anti-proliferative activity.²⁷ In this experiment, MCF-7, HeLa and NKE cells were treated with various concentrations (0 μM, 5 μM, 10 μM, 20 μM, 30 μM and 50 μM) of the C-glycoside derivatives and the cell viability was measured by MTT assay.²⁸ The results are summarized in Table 2 and expressed in terms of IC₅₀ values (half-maximal inhibitory concentration).

Out of 28 tested compounds, compound 8 (IC₅₀: 17.77 ± 1.46 μM), compound 24 (IC₅₀: 9.588 ± 5.25 μM) and compound 28 (IC₅₀: 22.98 ± 4.19 μM) showed higher efficacy than etoposide (IC₅₀: 24.77 ± 3.4 μM) in MCF-7 cells. In another set of HeLa cells, compound 8 (IC₅₀: 15.98 ± 1.85 μM), compound 24 (IC₅₀: 14.22 ± 3.94 μM), compound 28 (IC₅₀: 22.29 ± 4.03 μM) and also compound 9 (IC₅₀: 10.93 ± 5.36 μM) exerted lower IC₅₀ as compared to etoposide (IC₅₀: 29.12 ± 8.56 μM). From these results, it was found that compound 24 was more potent in both MCF-7 and HeLa cells as compared to compound 8 and compound 28, whereas compound 9 was only effective in HeLa cells. Consequently, an evaluation of the cytotoxic effect of compounds 8, 24 and 28 against NKE cells showed significantly higher IC₅₀ values than their IC₅₀ values against MCF-7 and HeLa cells [compound 24 (IC₅₀: 54.14 ± 1.56 μM), compound 8 (IC₅₀: 46.03 ± 10.56 μM) and compound 28 (IC₅₀: 40.29 ± 11.87 μM)]. Subsequently, when the selectivity indexes (SI) were calculated based on the IC₅₀ value ratio of compounds in NKE vs. MCF-7, it was found that compound 24 (SI = 7.93 ± 4.18) possessed higher SI in comparison to compound 8 (SI = 2.56 ± 0.38) and compound 28 (SI = 4.05 ± 0.06). In the case of HeLa cells, compound 24 also showed a better SI value than the other two compounds [compound 24 (SI = 3.42 ± 0.30), 8 (SI = 3.33 ± 0.34) and 28 (SI = 1.71 ± 0.20)].

The activity of compound 24 was further tested against MDA-MB-231 cells. Interestingly, it was also observed that compound 24 was very effective in inhibiting the proliferation of MDA-MB-231 cells and the calculated IC₅₀ value was 19.16 ± 5.97 μM (Fig. 1). Based on the preliminary MTT experimental data and selectivity index, compound 24 has been selected as a promising anti-cancer agent and considered for further evaluation.

Treatment with compound 24 exhibited a significant decrease in the cell reproductive death (colony formation) in MCF-7 cells. Clonogenic assay or colony formation assay,²⁹ an *in vitro* cell survival assay, was performed in MCF-7 cells using compound 24 to check the ability of a single cell to grow into a colony in the presence of different doses of the compound (0 nM, 100 nM, 500 nM, 1.25 μM, 2.5 μM and 5 μM) for 15 days. Interestingly, counting of colonies clearly demonstrated that compound 24 significantly decreased the colony numbers in MCF-7 cells even at a dose of 1.25 μM (Fig. 2). At a dose of 5 μM of compound 24, not a single colony was observed, as shown in Fig. 2.

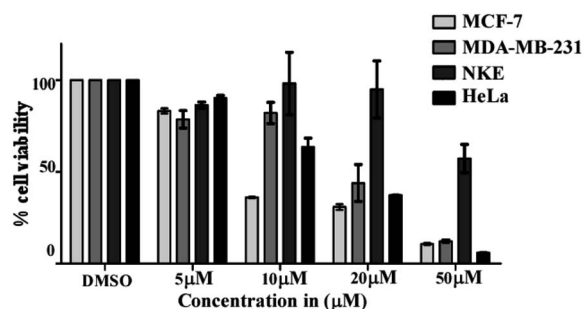


Fig. 1 Determination of dose-dependent cytotoxicity of compound 24 on various human cell lines.



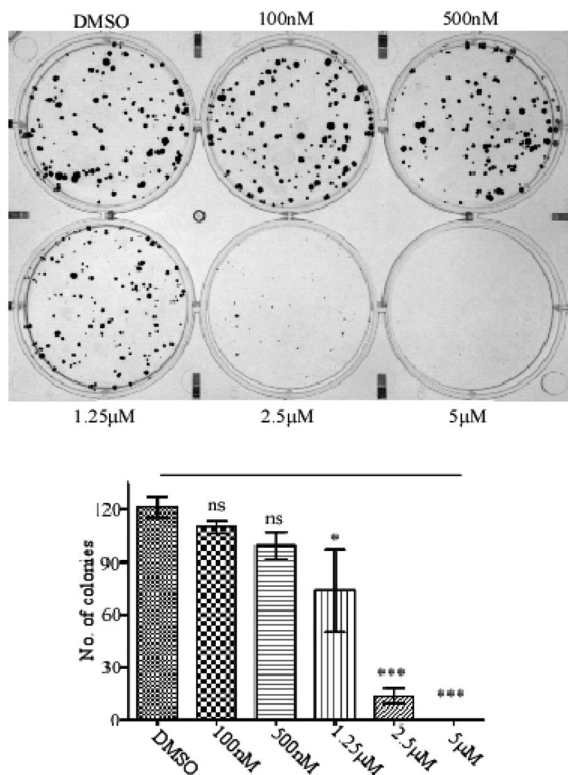


Fig. 2 Effects of compound 24 on colony formation on MCF-7 cells. The results show a minimum of three independent experiments and the bar graph shows mean \pm SEM (* p < 0.05, ** p < 0.01, *** p < 0.001, ns = not significant).

Compound 24 mediated G2/M arrest contributes to growth inhibition in MCF-7 cells. The cell cycle process has a critical role in the cellular growth process. In order to check whether compound 24 mediated inhibition of cancer cell proliferation has any relationship with cell cycle arrest, MCF-7 cells were treated with varying concentrations of compound 24 (0 μ M, 5 μ M, 10 μ M, 20 μ M and 30 μ M) for 24 h and stained with propidium iodide and the cell cycle profile checked in FACS. Interestingly, it was found that there was an increase in the percentage of cells in the G2/M phase of the cell cycle (19.4%, 29.6%, 34.2%, 37.1%, 45.2%), as is evident from Fig. 3. 2 μ M of nocodazole was used as a positive control³⁰ for G2/M arrest, where 81.7% cells were found in the G2/M phase. From this experiment it was confirmed that compound 24 mediated apoptosis G2/M arrest contributes to the growth inhibition in MCF-7 cells.

Treatments with compound 24 induced significant apoptosis in MCF-7 cells. Apoptosis is a crucial phenomenon which blocks the proliferation of cells.³¹ In order to check whether compound 24 mediated inhibition of cancer cell proliferation has any relationship with apoptosis, we treated MCF-7 cells with varying concentrations of compound 24 (0 μ M, 5 μ M, 10 μ M, 20 μ M and 30 μ M) and stained with annexin V-FITC/PI for analysis of apoptotic cells. Interestingly, $6.11 \pm 0.11\%$, $14.71 \pm 1.29\%$, $15.82 \pm 1.78\%$, $28.46 \pm 10.36\%$ cells were found to have annexin V-FITC⁺/PI⁻ and annexin V-FITC⁺/

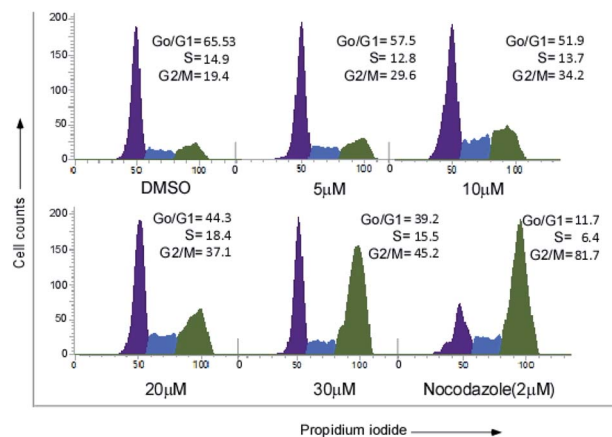


Fig. 3 Compound 24 induced G2/M phase cell cycle arrest in MCF-7 cells.

PI⁺ when treated with concentrations of 5 μ M, 10 μ M, 20 μ M, 30 μ M, respectively, in comparison with a vehicle control ($4.22 \pm 0.45\%$). Thus, the apoptotic potential of compound 24 has been established by this experiment. MCF-7 cells were shown to be prone to apoptosis in a dose-dependent manner except for doses of 10 μ M and 20 μ M, where a dose-dependent increase in

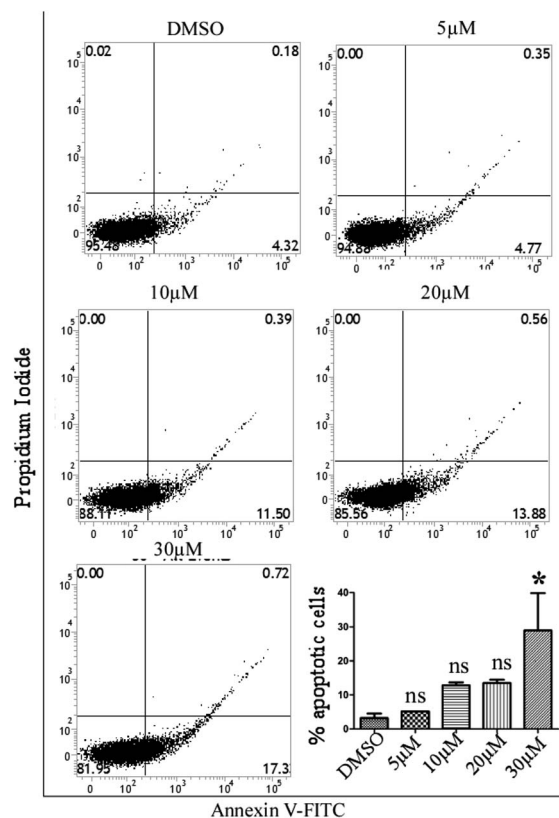


Fig. 4 Demonstrates the apoptosis-inducing ability of compound 24 in the MCF-7 cell line. After treatment with compound 24, the MCF-7 cells were incubated with 5 μ l of annexin V-FITC and 5 μ l of PI for 15 min at room temperature and flow cytometric analysis was performed.



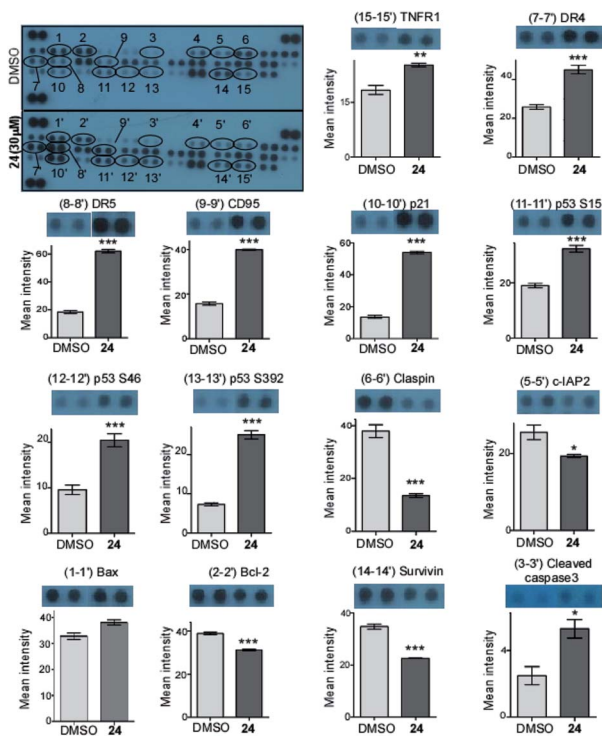


Fig. 5 The Human Apoptosis Array detects the expression of multiple apoptosis-related proteins in treated and untreated MCF-7 cells. The bar graphs indicate the band intensity. Data are representative of two independent experiments and bar graph shows mean \pm SEM ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ns = not significant).

apoptosis was not prominent. This significantly elevated level of apoptosis, confirmed through annexin V binding with translocated phosphatidylserine, provided a clue that the process of apoptosis definitely contributes to the compound **24** mediated cytotoxicity (Fig. 4).

Human apoptosis proteome profile array demonstrated compound **24 induced increase in pro-apoptotic and decrease in anti-apoptotic proteins in MCF-7 cell line.** Annexin V-FITC staining confirmed that apoptosis is one of the key processes contributing to the compound **24** mediated cytotoxicity. In order to gain a comprehensive idea regarding the molecular changes in the expression level of several proteins associated with apoptosis machinery, a Human Apoptosis Array kit (R&D Biosystem) was used. MCF-7 cells were treated with 30 μ M of compound **24** for 48 hours and lysates were used to profile the expression level of 35 proteins associated with the apoptosis machinery. Analysis of membranes indicated the differential expression of several apoptosis-related proteins, as shown in Fig. 5. Interestingly, the dot images of the Human Apoptosis Array kit showed significant down-regulation of several anti-apoptotic proteins, including Bcl-2, survivin, cIAP1, cIAP2 as well as claspain. In contrast, pro-apoptotic protein Bax and p21 were significantly up-regulated due to treatment with compound **24**. Since the Bax/Bcl-2 ratio plays a critical role in the apoptosis process, compound **24** probably mediates apoptosis by changing the Bax/Bcl-2 ratio. In addition, we also observed a significant increase in the expression of some

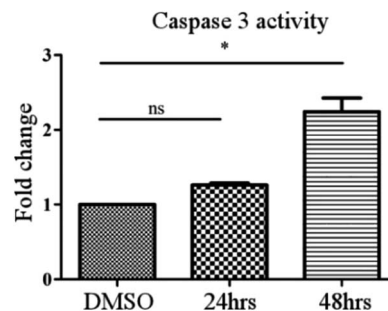


Fig. 6 Caspase-3 cleavage activity in MCF-7 cell extracts after treatment with compound **24**. Caspase-3 activity was measured by determining the ability of cell extracts to cleave the colorimetric substrate, Ac-DEVD-pNA, and plotted as a concentration in μ M of the cleaved pNA in the extract. Data represent the means \pm SEM of 2 independent experiments. $*P < 0.01$ and ns = non significant represent significant differences compared to the control condition.

proteins (TNFR1, CD4/TRAIL-1, CD5/TRAIL-2, CD95/Fas) that help in the receptor-mediated programmed cell death process. More importantly, a significant increase in the expression of cleaved caspase 3 was observed under treated conditions as compared to the control condition (Fig. 4). In addition, a human apoptosis proteome profile array using MCF-7 cells showed that treatment with compound **24** resulted in an increase in the expression of several pro-apoptotic proteins (Bax, p21, p53 etc.) and a decrease in the expression of anti-apoptotic proteins (survivin, claspain, bcl-2, etc.), as shown in Fig. 4. This imbalance between pro-apoptotic and anti-apoptotic proteins within the cells leads to apoptosis. It is well established that p53 phosphorylation at ser15 (DNA damage response)^{32,33} ser 46 (induction of apoptosis)^{34,35} and ser 392 (growth suppression)³⁶ plays an important role in cell proliferation. Interestingly, an increased level of total p53 along with p-p53 ser15, ser46, and ser392 was observed from proteome profiling and Western-immunoblotting analysis (Fig. 5). These findings suggested that both intrinsic as well as extrinsic apoptosis pathways contribute to compound **24** mediated apoptosis.

Caspase 3 activity assay. Since, caspase-3 activity is the key factor in apoptosis induction, it was decided to carry out a caspase-3/7 activity assay (colorimetric) using compound **24** to establish its potential to induce apoptosis.³⁷ In MCF-7 cells, a 2-fold increase in caspase activity was measured at a concentration of 30 μ M when compared with the control. Interestingly, such a level of caspase activation can be considered sufficient to cause cell death and the results clearly indicated that the cell death was induced by increased caspase-3 activities (Fig. 6).

Quantitative structure activity relationship analysis. In order to gain further information about the structure–function relationship of the active compounds, 3D-QSAR analyses of the active compounds were carried out using the Schrödinger program.³⁸ Model building was performed using partial least-squares regression (PLS).³⁹ The 3D-QSAR study described the pharmacophoric features of *C*-glycosylated cinnamoylfuran derivatives that contribute most towards the elicitation of an



Table 3 Statistical results for 3D-QSAR model^a

Training set	Test set
$N_1 = 18$	$N_2 = 9$
Corr. coefficient = 0.9407	Corr. coefficient = 0.8694
RMSD = 0.1995	RMSD = 0.2631
Max. fit value = 7.9529	Max. fit value = 3.9561
Weight = 1.1257	Weight = 1.1276

^a N_1 and N_2 : number of molecules in the training and test set, respectively; RMSD: root-mean square deviation.

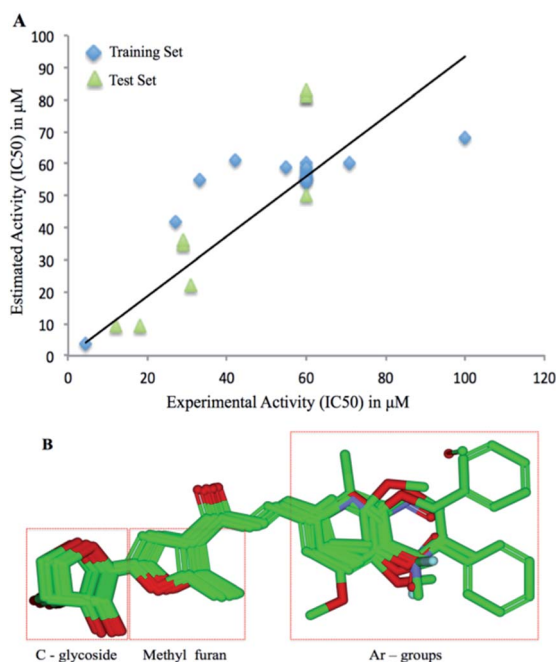


Fig. 7 3D-QSAR analysis of the compounds evaluated for anti-cancer activities. (A): Regression plot representing the statistical analysis with reference to the predicted IC_{50} value and theoretical IC_{50} values. (B): Alignment of the molecules showing conserved C-glycosylfuran moiety and variable aryl groups.

anti-cancer property. To analyze the anti-cancer property of the selected data set compounds (28 molecules) by 3D-QSAR analyses, they were categorized into a training set (19 molecules) and a test set (9 molecules). The statistical significance of this model is given in Table 3. The training set molecules exhibited a higher fit value and correlation coefficient and a lower RMSD value, which denote the excellence of accommodating the active groups of the molecules in the pharmacophoric features. The correlation between the experimental IC_{50} and the estimated IC_{50} values of the training set and the test set molecules are shown in Fig. 7A. The alignment of the molecular conformations confirmed that the C-glycosylated furan moiety present in all molecules is the essential feature eliciting an anti-cancer response (Fig. 7B). Mapping of the most active compounds 24 and 8 with the pharmacophore model has shown excellent interaction with all pharmacophoric features. Whereas mapping of the least active compounds 15 and 19 did not

exhibit the interactions of their functional groups with all pharmacophoric features (Figure not shown). Substitution with an electron-donating alkyl group in the *meta*- and *para*- positions of the aromatic group (phenyl ring) increased the anti-cancer activity (compounds 8 and 24). The methyl group of the furan ring imparts hydrophobic interaction, which is also essential for improving affinity towards the target as an anti-cancer compound.

Conclusions

In summary, a series of C-glycosyl cinnamoylfuran derivatives were synthesized and evaluated for their *in vitro* cytotoxic activity against cancer cells (MCF-7 and HeLa) and normal cells (NKE). Three compounds (8, 24 and 28) showed significant cytotoxic effects on MCF-7 and HeLa cell lines. Based on the selectivity index, compound 24 was considered the most promising anticancer agent. Further biochemical studies showed that compound 24 mediated cytotoxicity appeared to be due to both intrinsic and extrinsic pathways of apoptosis. QSAR studies have also been carried out to establish the structure-activity relationships of the active compounds.

Experimental

General methods

All reactions were monitored by thin layer chromatography over silica gel coated TLC plates. The spots on TLC were visualized by warming ceric sulphate (2% $Ce(SO_4)_2$ in 2 N H_2SO_4) sprayed plates on a hot plate. Silica gel 230–400 mesh was used for column chromatography. 1H and ^{13}C NMR, 2D COSY, HSQC spectra were recorded on a Bruker Avance 500 MHz spectrometer using $CDCl_3$ as solvent and TMS as the internal reference unless stated otherwise. Chemical shift values are expressed in δ ppm. ESI-MS were recorded on a Micromass mass spectrometer. Elementary analysis was carried out on a Carlo Erba analyzer. Optical rotations were measured at 25 °C on a Jasco P-2000 polarimeter. Biological experiments were carried out in a Shimadzu UV-2401PC spectrophotometer. Commercially available grades of organic solvents of adequate purity were used in many reactions. The cell culture media along with ingredients were purchased from HiMedia (India). FBS was procured from Gibco (USA). DMSO was purchased from SRL (India). An annexin V-FITC apoptosis assay kit was bought from BD Bioscience (India). A Caspase 3 Assay Kit (ab39401) and Human Apoptosis Array Kit were purchased from AbCam (UK) and R&D Systems (USA), respectively. MCF-7, MDA-MB-231 (human breast cancer) cells, HeLa (human cervical cancer) cells were obtained from the central cell repository of National Center for Cell Science (NCCS), Pune, India. Normal Kidney Epithelial cell (NKE) was a kind gift from Dr K. Biswas, Bose Institute, Kolkata. The cell lines were cultured in RPMI-1640 or DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, non-essential amino acids, 100 units per ml penicillin, 50 $\mu g\ ml^{-1}$ streptomycin and 50 $\mu g\ ml^{-1}$ gentamicin sulfate at 37 °C in a humidified incubator containing 5% CO_2 atmosphere.



General experimental condition for the preparation of compound 7–34

To a solution of compound 4 or 5 or 6 (1 mmol) in CH₃OH (10 ml) were added aromatic aldehyde (1.05 mmol) and NaOCH₃ (100 mg) and the reaction mixture was allowed to stir at room temperature for 12 h. The reaction mixture was diluted with water and acidified with 1 N HCl. The white solid precipitated out from the solution, which was filtered and crystallized from EtOH to give pure products 7–34 in appropriate yield, as mentioned in the schemes.

Analytical data of the compounds 7–34

Compound 7. Yellow solid; *R*_f: 0.20 (hexane–EtOAc; 1 : 2); mp 120–122 °C [EtOH]; [α]_D²⁵ –51 (*c* 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 7.71 (d, 1H, *J* = 15.5 Hz, COCH = CH), 7.50 (d, 2H, *J* = 7.5 Hz, Ar-H), 7.21 (d, 2H, *J* = 7.5 Hz, Ar-H), 7.14 (d, 1H, *J* = 15.5 Hz, COCH = CH), 6.72 (s, 1H, H-4), 4.68 (d, 1H, *J* = 6.5 Hz, H-1'), 4.42–4.38 (m, 2H, H-4'), 4.29–4.26 (m, 1H, H-3'), 3.92 (dd, 1H, *J* = 3.0, 10.5 Hz, H-2'), 2.65 (s, 3H, CH₃), 2.41 (s, 3H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 185.7 (C=O), 159.7 (C-4), 150.9 (C-1), 143.5 (COCH = CH), 140.8, 132.2, 129.6, 128.4 (Ar-C), 122.6 (COCH = CH), 122.3 (C-2), 109.0 (C-3), 77.2 (C-1'), 74.8 (C-3'), 73.1 (C-4'), 71.0 (C-2'), 21.5, 14.6 (2C, CH₃); ESI-MS: 351.1 [M + Na]⁺; anal. calcd for C₁₉H₂₀O₅ (328.36): C, 69.50; H, 6.14%; found: C, 69.45; H, 6.34%.

Compound 8. Yellow solid; *R*_f: 0.22 (hexane–EtOAc; 1 : 2); mp 128–130 °C [EtOH]; [α]_D²⁵ –34 (*c* 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 7.58 (d, 1H, *J* = 16.0 Hz, COCH = CH), 7.10 (d, 1H, *J* = 7.5 Hz, Ar-H), 7.03 (s, 1H, Ar-H), 6.96 (d, 1H, *J* = 15.5 Hz, COCH = CH), 6.79 (d, 1H, *J* = 8.0 Hz, Ar-H), 6.65 (s, 1H, H-4), 4.61 (d, 1H, *J* = 6.0 Hz, H-1'), 4.32–4.29 (m, 2H, H-4'), 4.19–4.17 (m, 1H, H-3'), 3.86–3.82 (m, 7H, H-2', 2 OCH₃), 2.55 (s, 3H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 185.7 (C=O), 159.7 (C-4), 151.5 (C-1), 150.0, 149.2 (Ar-C), 143.8 (COCH = CH), 127.6 (Ar-C), 122.2 (COCH = CH), 122.3 (C-2), 121.5 (Ar-C), 110.1, 110.2 (Ar-C), 109.1 (C-3), 77.1 (C-1'), 74.7 (C-3'), 73.1 (C-4'), 71.0 (C-2'), 55.7 (2C, 2 OCH₃), 14.6 (CH₃); ESI-MS: 397.1 [M + Na]⁺; anal. calcd for C₂₀H₂₂O₇ (374.38): C, 64.16; H, 5.92%; found: C, 64.42; H, 6.01%.

Compound 9. Yellow solid; *R*_f: 0.31 (hexane–EtOAc; 1 : 2); mp 141–142 °C [EtOH]; [α]_D²⁵ –41 (*c* 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 7.63 (d, 1H, *J* = 15.5 Hz, COCH = CH), 6.05 (d, 1H, *J* = 15.5 Hz, COCH = CH), 6.81 (s, 2H, Ar-H), 6.73 (s, 1H, H-4), 4.69 (d, 1H, *J* = 7.0 Hz, H-1'), 4.43–4.38 (m, 2H, H-4'), 4.30 (dd, 1H, *J* = 5.0, 10.0 Hz, H-3'), 3.92–3.84 (m, 10H, H-2', 3 OCH₃), 2.66 (s, 3H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 185.4 (C=O), 159.8 (C-4), 153.4 (Ar-C), 149.9 (C-1), 143.6 (COCH = CH), 137.7 (Ar-C), 130.2 (C-2), 126.2 (Ar-C), 123.0 (COCH = CH), 109.0 (C-3), 105.7 (2C, Ar-C), 77.2 (C-1'), 74.7 (C-3'), 73.2 (C-4'), 71.0 (C-2'), 60.9, 56.1 (3C, 3OCH₃), 14.6 (CH₃); ESI-MS: 427.1 [M + Na]⁺; anal. calcd for C₂₁H₂₄O₈ (404.41): C, 62.37; H, 5.98%; found: C, 62.47; H, 6.11%.

Compound 10. Yellow solid; *R*_f: 0.28 (hexane–EtOAc; 1 : 2); mp 99–101 °C [EtOH]; [α]_D²⁵ –21 (*c* 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 7.72 (d, 1H, *J* = 15.5 Hz, COCH = CH), 7.56 (d, 2H, *J* = 9.0 Hz, Ar-H), 7.44–7.34 (m, 5H, Ar-H), 7.07 (d, 1H, *J* =

15.5 Hz, COCH = CH), 7.00 (d, 2H, *J* = 9.0 Hz, Ar-H), 6.72 (s, 1H, H-4), 5.12 (brs, 2H, PhCH₂), 4.69 (d, 1H, *J* = 6.5 Hz, H-1'), 4.43–4.38 (m, 2H, H-4'), 4.30–4.27 (m, 1H, H-3'), 3.93 (dd, 1H, *J* = 3.0, 10.5 Hz, H-2'), 2.65 (s, 3H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 185.7 (C=O), 160.7 (C-4), 150.2 (C-1), 143.2 (COCH = CH), 136.3, 130.1, 128.6 (Ar-C), 128.1 (COCH = CH), 127.7, 127.4 (Ar-C), 121.5 (C-2), 115.2 (Ar-C), 109.0 (C-3), 77.2 (C-1'), 74.8 (C-3'), 73.2 (C-4'), 71.0 (C-2'), 70.0 (PhCH₂), 14.6 (CH₃); ESI-MS: 443.11 [M + Na]⁺; anal. calcd for C₂₅H₂₄O₆ (420.45): C, 71.41; H, 5.75%; found: C, 71.30; H, 5.87%.

Compound 11. Yellow solid; *R*_f: 0.24 (hexane–EtOAc; 1 : 2); mp 124–125 °C [EtOH]; [α]_D²⁵ –15 (*c* 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 7.56 (d, 1H, *J* = 15.5 Hz, COCH = CH), 7.43 (d, 2H, *J* = 8.0 Hz, Ar-H), 7.28 (d, 2H, *J* = 8.0 Hz, Ar-H), 7.04 (d, 1H, *J* = 15.5 Hz, COCH = CH), 6.62 (s, 1H, H-3), 4.58 (d, 1H, *J* = 6.5 Hz, H-1'), 4.31–4.27 (m, 2H, H-4'), 4.18–4.15 (m, 1H, H-3'), 3.81 (dd, 1H, *J* = 2.5, 10.0 Hz, H-2'), 2.54 (s, 3H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 186.3 (C=O), 161.1 (C-4), 150.0 (C-1), 141.9 (COCH = CH), 136.2, 133.2, 129.5, 128.4, 129.2 (Ar-C), 124.0 (COCH = CH), 122.2 (C-2), 108.9 (C-3), 77.1 (C-1'), 74.8 (C-3'), 73.1 (C-4'), 71.0 (C-2'), 14.6 (CH₃); ESI-MS: 371.0 [M + Na]⁺; anal. calcd for C₁₈H₁₇ClO₅ (348.78): C, 61.99; H, 4.91%; found: C, 61.84; H, 5.02%.

Compound 12. Yellow solid; *R*_f: 0.35 (hexane–EtOAc; 1 : 3); mp 122–123 °C [EtOH]; [α]_D²⁵ –10 (*c* 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 8.54 (d, 1H, *J* = 15.5 Hz, COCH = CH), 8.21 (d, 1H, *J* = 8.0 Hz, Ar-H), 7.86–7.76 (m, 2H, Ar-H), 7.55–7.42 (m, 4H, Ar-H), 7.21 (d, 1H, *J* = 15.5 Hz, COCH = CH), 6.71 (s, 1H, H-4), 4.67 (d, 1H, *J* = 6.0 Hz, H-1'), 4.35–4.34 (m, 2H, H-4'), 4.22–4.19 (m, 1H, H-3'), 3.87–3.85 (m, 1H, H-2'), 2.63 (s, 3H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 185.2 (C=O), 160.0 (C-4), 150.3 (C-1), 140.2 (COCH = CH), 133.7, 132.1, 131.8, 130.7, 128.7, 127.5, 126.4, 125.3, 125.1, (Ar-C), 123.5 (COCH = CH), 122.3 (C-2), 108.9 (C-3), 77.1 (C-1'), 74.8 (C-3'), 73.2 (C-4'), 71.0 (C-2'), 14.7 (CH₃); ESI-MS: 387.1 [M + Na]⁺; anal. calcd for C₂₂H₂₀O₅ (364.39): C, 72.51; H, 5.53%; found: C, 72.42; H, 5.65%.

Compound 13. Yellow solid; *R*_f: 0.15 (hexane–EtOAc; 1 : 1); mp 103–104 °C [EtOH]; [α]_D²⁵ –26 (*c* 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 7.60 (d, 1H, *J* = 9.0 Hz, Ar-H), 7.58 (d, 1H, *J* = 15.5 Hz, COCH = CH), 7.18 (d, 1H, *J* = 15.5 Hz, COCH = CH), 6.81–6.79 (m, 2H, Ar-H), 6.60 (s, 1H, H-4), 4.77 (d, 1H, *J* = 7.0 Hz, H-1'), 4.52–4.46 (m, 2H, H-4'), 4.38–4.35 (m, 1H, H-3'), 4.01–3.98 (m, 1H, H-2'), 2.75 (s, 3H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 185.2 (C=O), 160.0 (C-4), 150.3 (C-1), 144.7 (COCH = CH), 129.3 (C-2), 121.2 (COCH = CH), 116.0, 112.6 (Ar-C), 109.0 (C-3), 77.2 (C-1'), 74.7 (C-3'), 73.1 (C-4'), 71.0 (C-2'), 14.6 (CH₃); ESI-MS: 327.0 [M + Na]⁺; anal. calcd for C₁₆H₁₆O₆ (304.29): C, 63.15; H, 5.30%; found: C, 63.10; H, 5.42%.

Compound 14. Yellow solid; *R*_f: 0.33 (hexane–EtOAc; 1 : 3); mp 93–95 °C [EtOH]; [α]_D²⁵ –37 (*c* 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 7.76 (d, 1H, *J* = 15.5 Hz, COCH = CH), 7.58 (d, 2H, *J* = 9.0 Hz, Ar-H), 7.05 (d, 1H, *J* = 15.5 Hz, COCH = CH), 6.78–6.75 (m, 3H, Ar-H), 6.66 (s, 1H, H-4), 4.73 (d, 1H, *J* = 7.0 Hz, H-1'), 4.50–4.44 (m, 2H, H-4'), 4.37–4.32 (m, 1H, H-3'), 3.99 (dd, 1H, *J* = 3.0, 10.0 Hz, H-2'), 3.15 (s, 6H, N (CH₃)₂), 2.46 (s, 3H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 185.7 (C=O), 159.7 (C-4), 149.7 (C-1), 148.6 (Ar-C), 144.7 (COCH = CH), 130.4 (2C, Ar-C),



122.6 (COCH = CH), 121.9 (Ar-C), 118.5 (C-2), 111.9 (2C, Ar-C), 109.0 (C-3), 77.1 (C-1'), 74.7 (C-3'), 73.2 (C-4'), 71.1 (C-2'), 40.1 (2C, N (CH₃)₂), 14.6 (2C, CH₃); ESI-MS: 380.1 [M + Na]⁺; anal. calcd for C₂₀H₂₃NO₅ (357.40): C, 67.21; H, 6.49%; found: C, 67.14; H, 6.60%.

Compound 15. Yellow solid; *R*_f: 0.25 (hexane–EtOAc; 1 : 2); mp 102–104 °C [EtOH]; [α]_D²⁵ –20 (c 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 7.80 (d, 1H, *J* = 16.0 Hz, COCH = CH), 7.69–7.68 (m, 2H, Ar-H), 7.50–7.48 (m, 3H, Ar-H), 7.27 (d, 1H, *J* = 16.0 Hz, COCH = CH), 6.81 (s, 1H, H-4), 4.78 (d, 1H, *J* = 6.5 Hz, H-1'), 4.51–4.47 (m, 2H, H-4'), 4.38–4.35 (m, 1H, H-3'), 4.01 (dd, 1H, *J* = 3.0, 10.0 Hz, H-2'), 2.73 (s, 3H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 185.5 (C=O), 159.9 (C-4), 150.0 (C-1), 143.5 (COCH = CH), 134.7, 130.4, 128.9, 128.4 (Ar-C), 123.6 (COCH = CH), 122.3 (C-2), 109.0 (C-3), 77.2 (C-1'), 74.8 (C-3'), 73.2 (C-4'), 71.0 (C-2'), 14.6 (CH₃); ESI-MS: 337.1 [M + Na]⁺; anal. calcd for C₁₈H₁₈O₅ (314.33): C, 68.78; H, 5.77%; found: C, 68.65; H, 5.90%.

Compound 16. Yellow solid; *R*_f: 0.30 (hexane–EtOAc; 1 : 3); mp 108–110 °C [EtOH]; [α]_D²⁵ –21 (c 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 13.2 (s, 1H, NH), 6.76 (d, 1H, *J* = 14.5 Hz, COCH = CH), 6.75 (d, 1H, *J* = 8.0 Hz, Ar-H), 6.67 (s, 1H, H-4), 6.53–6.49 (m, 3H, COCH = CH, Ar-H), 4.77 (d, 1H, *J* = 6.5 Hz, H-1'), 4.48–4.46 (m, 2H, H-4'), 4.37–4.34 (m, 1H, H-3'), 3.98 (dd, 1H, *J* = 2.5, 10.5 Hz, H-2'), 2.65 (s, 3H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 185.2 (C=O), 157.1 (C-4), 149.0 (C-1), 138.9 (C-2), 128.5 (COCH = CH), 123.9 (Ar-C), 118.9 (COCH = CH), 111.4 (Ar-C), 109.9 (C-3), 107.7, 103.2 (Ar-C), 77.3 (C-1'), 74.6 (C-3'), 73.0 (C-4'), 71.0 (C-2'), 14.0 (CH₃); ESI-MS: 326.1 [M + Na]⁺; anal. calcd for C₁₆H₁₇NO₅ (303.31): C, 63.36; H, 5.65%; found: C, 63.20; H, 5.48%.

Compound 17. Pale yellow solid; *R*_f: 0.30 (hexane–EtOAc; 2 : 5); mp 145–147 °C [EtOH]; [α]_D²⁵ –32 (c 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 8.26 (d, 2H, *J* = 6.5 Hz, Ar-H), 7.75 (d, 1H, *J* = 16.0 Hz, COCH = CH), 7.73 (d, 2H, *J* = 7.5 Hz, Ar-H), 7.29 (d, 1H, *J* = 16.0 Hz, COCH = CH), 6.76 (s, 1H, H-3), 4.71 (d, 1H, *J* = 7.0 Hz, H-1'), 4.46–4.41 (m, 2H, H-4'), 4.32–4.22 (m, 1H, H-3'), 3.94 (dd, 1H, *J* = 3.0, 10.0 Hz, H-2'), 2.65 (s, 3H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 185.7 (C=O), 159.7 (C-4), 142.1 (C-1), 140.1 (COCH = CH), 132.2, 129.6, 128.4 (Ar-C), 127.4 (COCH = CH), 124.2 (Ar-C), 123.1 (C-2), 108.8 (C-3), 77.2 (C-1'), 74.8 (C-3'), 73.3 (C-4'), 71.0 (C-2'), 14.7 (CH₃); ESI-MS: 382.1 [M + Na]⁺; anal. calcd for C₁₈H₁₇NO₇ (359.33): C, 60.17; H, 4.77%; found: C, 60.00; H, 4.95%.

Compound 18. Yellow solid; *R*_f: 0.28 (hexane–EtOAc; 1 : 2); mp 133–135 °C [EtOH]; [α]_D²⁵ –35 (c 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 7.59 (d, 1H, *J* = 16.5 Hz, COCH = CH), 7.45 (d, 2H, *J* = 8.5 Hz, Ar-H), 6.95 (d, 1H, *J* = 15.5 Hz, COCH = CH), 6.82 (d, 2H, *J* = 8.0 Hz, Ar-H), 6.61 (s, 1H, H-3), 4.59 (d, 1H, *J* = 6.5 Hz, H-1'), 4.32–4.28 (m, 2H, H-4'), 4.19 (dd, 1H, *J* = 4.5, 10.0 Hz, H-3'), 3.82 (dd, 1H, *J* = 3.0, 10.0 Hz, H-2'), 3.75 (s, 3H, CH₃), 2.53 (s, 3H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 185.2 (C=O), 161.2 (Ar-C), 159.0 (C-4), 151.1 (C-1), 143.3 (COCH = CH), 130.1, 127.4 (Ar-C), 123.5 (C-2), 121.4 (COCH = CH), 114.4 (Ar-C), 109.1 (C-3), 77.2 (C-1'), 74.7 (C-3'), 73.2 (C-4'), 71.0 (C-2'), 55.3 (OCH₃), 14.7 (CH₃); ESI-MS: 367.1 [M + Na]⁺; anal. calcd for C₁₉H₂₀O₆ (344.36): C, 66.27; H, 5.85%; found: C, 66.07; H, 6.00%.

Compound 19. Yellow solid; *R*_f: 0.32 (hexane–EtOAc; 1 : 3); mp 138–140 °C [EtOH]; [α]_D²⁵ –31 (c 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 7.57 (d, 1H, *J* = 15.5 Hz, COCH = CH), 7.43 (d, 2H, *J* = 8.5 Hz, Ar-H), 6.93 (d, 1H, *J* = 15.5 Hz, COCH = CH), 6.70 (d, 2H, *J* = 8.0 Hz, Ar-H), 6.60 (s, 1H, H-3), 4.58 (d, 1H, *J* = 6.0 Hz, H-1'), 4.32–4.27 (m, 2H, H-4'), 4.17–4.15 (dd, 1H, *J* = 4.5, 10.0 Hz, H-3'), 3.99 (q, 2H, *J* = 7.0 Hz each, OCH₂CH₃), 3.81 (dd, 1H, *J* = 3.0, 10.0 Hz, H-2'), 2.52 (s, 3H, CH₃), 1.36 (t, 3H, *J* = 7.0 Hz, OCH₂CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 185.7 (C=O), 161.0 (Ar-C), 159.6 (C-4), 149.8 (C-1), 143.5 (COCH = CH), 130.2, 127.2 (Ar-C), 122.4 (C-2), 121.2 (COCH = CH), 114.8 (Ar-C), 109.1 (C-3), 77.1 (C-1'), 74.7 (C-3'), 73.1 (C-4'), 71.0 (C-2'), 63.5 (OCH₂CH₃), 14.7 (CH₃), 14.6 (OCH₂CH₃); ESI-MS: 381.1 [M + Na]⁺; anal. calcd for C₂₀H₂₂O₆ (358.39): C, 67.03; H, 6.19%; found: C, 66.90; H, 6.27%.

Compound 20. Yellow solid; *R*_f: 0.24 (hexane–EtOAc; 1 : 2); mp 125–127 °C [EtOH]; [α]_D²⁵ –40 (c 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 7.56 (d, 1H, *J* = 16.0 Hz, COCH = CH), 7.21–7.15 (m, 1H, Ar-H), 7.07–6.98 (m, 3H, COCH = CH, Ar-H), 6.83 (d, 1H, *J* = 8.0 Hz, Ar-H), 6.62 (s, 1H, H-3), 4.58 (d, 1H, *J* = 6.5 Hz, H-1'), 4.28–4.26 (m, 2H, H-4'), 4.16–4.12 (m, 1H, H-3'), 3.80–3.73 (m, 4H, OCH₃, H-2'), 2.50 (s, 3H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 185.7 (C=O), 159.9 (Ar-C), 159.9 (C-4), 150.0 (C-1), 143.5 (COCH = CH), 136.0, 129.9, 123.9 (Ar-C), 122.2 (C-2), 121.0 (COCH = CH), 116.2, 113.4 (Ar-C), 109.1 (C-3), 77.1 (C-1'), 74.8 (C-3'), 73.1 (C-4'), 71.0 (C-2'), 55.2 (OCH₃), 14.6 (CH₃); ESI-MS: 367.1 [M + Na]⁺; anal. calcd for C₁₉H₂₀O₆ (344.36): C, 66.27; H, 5.85%; found: C, 66.10; H, 5.99%.

Compound 21. Yellow solid; *R*_f: 0.27 (hexane–EtOAc; 1 : 2); mp 155–157 °C [EtOH]; [α]_D²⁵ –16 (c 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 8.12 (d, 1H, *J* = 16.0 Hz, COCH = CH), 7.69 (dd, 1H, *J* = 2.0, 7.0 Hz, Ar-H), 7.44 (dd, 1H, *J* = 1.0, 7.5 Hz, Ar-H), 7.34–7.24 (m, 2H, Ar-H), 7.16 (d, 1H, *J* = 16.0 Hz, COCH = CH), 6.74 (s, 1H, H-3), 4.71 (d, 1H, *J* = 6.5 Hz, H-1'), 4.45–4.39 (m, 2H, H-4'), 4.30–4.27 (m, 1H, H-3'), 3.92 (dd, 1H, *J* = 3.0, 10.0 Hz, H-2'), 2.65 (s, 3H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 186.2 (C=O), 160.3 (C-4), 149.9 (C-1), 139.3 (COCH = CH), 135.5, 133.1, 131.1, 130.3, 127.7, 127.0 (Ar-C), 126.4 (COCH = CH), 122.1 (C-2), 109.1 (C-3), 77.2 (C-1'), 74.8 (C-3'), 73.3 (C-4'), 71.0 (C-2'), 14.7 (CH₃); ESI-MS: 371.0 [M + Na]⁺; anal. calcd for C₁₈H₁₇ClO₅ (348.78): C, 61.99; H, 4.91%; found: C, 61.82; H, 5.05%.

Compound 22. Yellow solid; *R*_f: 0.18 (hexane–EtOAc; 1 : 2); mp 121–123 °C [EtOH]; [α]_D²⁵ –18 (c 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 7.61–7.39 (m, 5H, *J* = 16.0 Hz, COCH = CH, Ar-H), 7.13 (d, 1H, *J* = 15.5 Hz, COCH = CH), 6.64 (s, 1H, H-3), 4.59 (d, 1H, *J* = 6.5 Hz, H-1'), 4.31–4.27 (m, 2H, H-4'), 4.18–4.15 (m, 1H, H-3'), 3.81 (dd, 1H, *J* = 3.0, 9.0 Hz, H-2'), 2.54 (s, 3H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 185.0 (C=O), 160.3 (C-4), 150.2 (C-1), 141.4 (COCH = CH), 138.1, 128.4, 126.9, 125.8 (Ar-C), 125.8 (COCH = CH), 122.2 (C-2), 108.9 (C-3), 77.1 (C-1'), 74.8 (C-3'), 73.2 (C-4'), 71.0 (C-2'), 14.7 (CH₃); ESI-MS: 405.1 [M + Na]⁺; anal. calcd for C₁₉H₁₇F₃O₅ (382.33): C, 59.69; H, 4.48%; found: C, 59.58; H, 4.60%.

Compound 23. Yellow solid; *R*_f: 0.27 (hexane–EtOAc; 1 : 2); mp 167–169 °C [EtOH]; [α]_D²⁵ –29 (c 1.0, CHCl₃); ¹H NMR (500



MHz, CDCl₃): δ 8.35 (s, 1H, Ar-H), 8.12 (d, 1H, J = 8.5 Hz, Ar-H), 7.76 (d, 1H, J = 7.5 Hz, Ar-H), 7.61 (d, 1H, J = 15.5 Hz, COCH = CH), 7.50 (t, 1H, J = 8.0 Hz each, Ar-H), 7.19 (d, 1H, J = 15.5 Hz, COCH = CH), 6.67 (s, 1H, H-3), 4.60 (d, 1H, J = 6.5 Hz, H-1'), 4.31–4.28 (m, 2H, H-4'), 4.18–4.15 (m, 1H, H-3'), 3.82 (dd, 1H, J = 2.5, 10.5 Hz, H-2'), 2.54 (s, 3H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 184.6 (C=O), 160.6 (C-4), 150.3 (C-1), 148.7 (Ar-C), 140.2 (COCH = CH), 136.5, 134.3, 129.9, 126.2, 124.5 (Ar-C), 122.2 (COCH = CH), 122.0 (C-2), 108.8 (C-3), 77.0 (C-1'), 74.8 (C-3'), 73.2 (C-4'), 71.0 (C-2'), 14.7 (CH₃); ESI-MS: 382.1 [M + Na]⁺; anal. calcd for C₁₈H₁₇NO₇ (359.33): C, 60.17; H, 4.77%; found: C, 60.05; H, 4.95%.

Compound 24. Yellow solid; R_f : 0.33 (hexane–EtOAc; 1 : 2); mp 98–100 °C [EtOH]; [α]_D²⁵ –21 (c 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 7.59 (d, 1H, J = 15.5 Hz, COCH = CH), 7.46–7.12 (m, 5H, Ar-H), 7.15–7.12 (m, 2H, Ar-H), 6.91 (d, 1H, J = 15.5 Hz, COCH = CH), 6.86 (d, 2H, J = 8.5 Hz, Ar-H), 6.66 (s, 1H, H-4), 5.16 (br s, 2H, PhCH₂), 4.67 (d, 1H, J = 6.0 Hz, H-1'), 4.37–4.35 (m, 2H, H-4'), 4.25–4.23 (m, 1H, H-3'), 3.90–3.88 (m, 4H, H-2', OCH₃), 2.58 (s, 3H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 185.7 (C=O), 159.6 (C-4), 152.1 (C-1), 150.0, 148.3 (Ar-C), 143.2 (COCH = CH), 136.7, 128.6, 128.0, 127.5, 127.3, (Ar-C), 123.3 (COCH = CH), 122.4 (Ar-C), 121.5 (C-2), 113.1, 111.5 (Ar-C), 109.1 (C-3), 77.1 (C-1'), 74.7 (C-3'), 73.2 (C-4'), 71.2 (PhCH₂), 71.0 (C-2'), 55.9 (OCH₃), 14.6 (CH₃); ESI-MS: 473.1 [M + Na]⁺; anal. calcd for C₂₆H₂₆O₇ (450.48): C, 69.32; H, 5.82%; found: C, 69.18; H, 6.00%.

Compound 25. White solid; R_f : 0.40 (hexane–EtOAc; 1 : 1); mp 113–115 °C [EtOH]; [α]_D²⁵ –68 (c 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 8.57 (s, 1H, Ar-H), 8.36 (dd, 1H, J = 1.5, 8.5 Hz, Ar-H), 7.99 (d, 1H, J = 7.5 Hz, Ar-H), 7.86 (d, 1H, J = 15.5 Hz, COCH = CH), 7.73 (t, 1H, J = 8.0 Hz each, Ar-H), 7.39 (d, 1H, J = 15.5 Hz, COCH = CH), 6.88 (s, 1H, H-3), 5.64–5.58 (m, 2H, H-3', H-2'), 5.03 (d, 1H, J = 6.5 Hz, H-1'), 4.51 (dd, 1H, J = 5.0, 10.5 Hz, H-4'_a), 4.09 (dd, 1H, J = 3.5, 10.5 Hz, H-4'_b), 2.79 (s, 3H, CH₃), 2.24, 2.22 (2 s, 6H, CH₃CO); ¹³C NMR (125 MHz, CDCl₃): δ 184.3 (C=O), 169.7, 169.5 (CH₃CO), 160.9 (C-4), 148.8 (C-1), 148.7 (Ar-C), 140.2 (COCH = CH), 136.6, 134.1, 129.9, 126.2, 124.5 (Ar-C), 122.2 (COCH = CH), 122.0 (C-2), 109.2 (C-3), 74.9 (C-1'), 73.7 (C-3'), 71.2 (C-2'), 70.7 (C-4'), 20.6, 20.5 (CH₃CO), 14.7 (CH₃); ESI-MS: 466.1 [M + Na]⁺; anal. calcd for C₂₂H₂₁NO₉ (443.40): C, 59.59; H, 4.77%; found: C, 59.47; H, 4.88%.

Compound 26. Yellow oil; R_f : 0.25 (hexane–EtOAc; 2 : 1); [α]_D²⁵ –20 (c 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 7.73 (d, 1H, J = 15.5 Hz, COCH = CH), 7.58 (d, 2H, J = 7.0 Hz, Ar-H), 7.51 (t, 2H, J = 7.5 Hz each, Ar-H), 7.44 (d, 1H, J = 7.0 Hz, Ar-H), 7.30 (dd, 1H, J = 1.5, 8.0 Hz, Ar-H), 7.24 (d, 1H, J = 1.5 Hz, Ar-H), 7.04 (d, 1H, J = 15.5 Hz, COCH = CH), 7.01 (d, 1H, J = 8.5 Hz, Ar-H), 6.81 (s, 1H, H-4), 5.63–5.58 (m, 2H, H-2', H-3'), 5.30 (brs, 2H, PhCH₂), 5.02 (d, 1H, J = 6.5 Hz, H-1'), 4.50 (dd, 1H, J = 5.0, 10.0 Hz, H-4'), 4.08 (dd, 1H, J = 3.5, 10.0 Hz, H-4'), 4.04 (s, 3H, OCH₃), 2.74 (s, 3H, CH₃), 2.24, 2.19 (2 s, 6H, CH₃CO); ¹³C NMR (125 MHz, CDCl₃): δ 185.4 (C=O), 169.7, 169.5 (2C, CH₃CO), 159.8 (C-4), 152.2 (C-1), 148.3 (Ar-C), 143.2 (COCH = CH), 136.7, 128.6, 128.0, 127.6, 127.3, (Ar-C), 123.4 (COCH = CH), 122.5 (C-2), 121.6, 113.1, 111.5 (Ar-C), 109.5 (C-3), 74.9 (C-1'), 73.7 (C-2'), 71.3 (C-3'), 71.1 (C-4'), 70.7 (PhCH₂), 55.9 (OCH₃), 20.6, 20.5 (2C,

CH₃CO), 14.6 (CH₃); ESI-MS: 557.1 [M + Na]⁺; anal. calcd for C₃₀H₃₀O₉ (534.55): C, 67.41; H, 5.66%; found: C, 67.30; H, 5.80%.

Compound 27. Yellow solid; R_f : 0.26 (hexane–EtOAc; 1 : 3); mp 123–125 °C [EtOH]; [α]_D²⁵ +17 (c 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 8.46 (s, 1H, Ar-H), 8.25 (d, 1H, J = 7.5 Hz, Ar-H), 7.89 (d, 1H, J = 8.0 Hz, Ar-H), 7.76 (d, 1H, J = 15.5 Hz, COCH = CH), 7.62 (t, 1H, J = 8.0 Hz each, Ar-H), 7.32 (d, 1H, J = 15.5 Hz, COCH = CH), 6.87 (s, 1H, H-3), 5.15 (d, 1H, J = 3.0 Hz, H-1'), 4.50 (brs, 1H, H-2'), 4.42–4.38 (m, 1H, H-4'_a), 4.26 (brs, 1H, H-4'_b), 3.87 (d, 1H, J = 10.0 Hz, H-2'), 2.67 (s, 3H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 184.7 (C=O), 160.5 (C-4), 148.7 (C-1), 148.4 (Ar-C), 140.3 (COCH = CH), 136.5, 134.2, 123.0, 126.3, 124.6 (Ar-C), 122.3 (COCH = CH), 122.1 (C-2), 109.2 (C-3), 78.0 (C-1'), 77.2 (C-2'), 76.8 (C-3'), 73.9 (C-4'), 14.6 (CH₃); ESI-MS: 382.1 [M + Na]⁺; anal. calcd for C₁₈H₁₇NO₇ (359.33): C, 60.17; H, 4.77%; found: C, 60.05; H, 4.95%.

Compound 28. Yellow solid; R_f : 0.18 (hexane–EtOAc; 1 : 1); mp 131–133 °C [EtOH]; [α]_D²⁵ +12 (c 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 7.70 (d, 1H, J = 15.5 Hz, COCH = CH), 7.59–7.41 (m, 5H, Ar-H), 7.27 (d, 1H, J = 8.0 Hz, Ar-H), 7.23 (d, 1H, J = 1.5 Hz, Ar-H), 7.05 (d, 1H, J = 15.0 Hz, COCH = CH), 6.98 (d, 1H, J = 8.5 Hz, Ar-H), 6.78 (s, 1H, H-3), 5.28 (brs, 2H, PhCH₂), 4.78 (d, 1H, J = 4.5 Hz, H-1'), 4.50–4.46 (m, 2H, H-3', H-2'), 4.23 (dd, 1H, J = 4.5, 10.0 Hz, H-4'_b), 4.10 (dd, 1H, J = 2.5, 10.0 Hz, H-4'_a), 4.02 (s, 3H, OCH₃), 2.71 (s, 3H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 185.6 (C=O), 159.4 (C-4), 152.1 (C-1), 148.3 (Ar-C), 143.6 (COCH = CH), 136.7, 128.6, 128.0, 127.3 (Ar-C), 123.6 (COCH = CH), 122.5 (C-2), 121.5, 113.1, 113.0, 111.5, 109.5 (Ar-C), 108.5 (C-3), 81.2 (C-1'), 80.1 (C-2'), 78.2 (C-3'), 73.5 (C-4'), 71.2 (PhCH₂), 55.9 (OCH₃), 14.6 (CH₃); ESI-MS: 473.1 [M + Na]⁺; anal. calcd for C₂₆H₂₆O₇ (450.48): C, 69.32; H, 5.82%; found: C, 69.20; H, 5.95%.

Compound 29. Yellow oil; R_f : 0.40 (hexane–EtOAc; 1 : 1); [α]_D²⁵ +26 (c 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 8.58 (s, 1H, Ar-H), 8.57 (dd, 1H, J = 1.0, 8.0 Hz, Ar-H), 7.99 (dd, 1H, J = 3.0, 7.5 Hz, Ar-H), 7.87 (d, 1H, J = 15.5 Hz, COCH = CH), 7.73 (t, 1H, J = 7.5, 8.0 Hz, Ar-H), 7.40 (d, 1H, J = 15.5 Hz, COCH = CH), 6.90 (s, 1H, H-3), 5.57 (d, 1H, J = 3.0 Hz, H-2'), 5.36–5.34 (m, 1H, H-3'), 4.91 (d, 1H, J = 4.0 Hz, H-1'), 4.33 (dd, 1H, J = 5.0, 10.5 Hz, H-4'_a), 4.20 (dd, 1H, J = 2.0, 11.5 Hz, H-4'_b), 2.77 (s, 3H, CH₃), 2.24, 2.23 (2 s, 6H, CH₃CO); ¹³C NMR (125 MHz, CDCl₃): δ 184.7 (C=O), 158.7 (C-4), 149.8 (C-1), 140.2 (COCH = CH), 136.8, 134.2, 129.9, 126.3, 124.5 (Ar-C), 122.2 (COCH = CH), 122.0 (C-2), 109.2 (C-3), 108.7 (Ar-C), 79.7 (C-1'), 78.4 (C-2'), 77.9 (C-3'), 72.0 (C-4'), 20.8, 20.5 (2C, CH₃CO), 14.7 (CH₃); ESI-MS: 466.1 [M + Na]⁺; anal. calcd for C₂₂H₂₁NO₉ (443.40): C, 59.59; H, 4.77%; found: C, 59.45; H, 4.90%.

Compound 30. Yellow oil; R_f : 0.23 (hexane–EtOAc; 1 : 3); [α]_D²⁵ +29 (c 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 7.73 (d, 1H, J = 15.5 Hz, COCH = CH), 7.58–7.23 (m, 7H, Ar-H), 7.05 (d, 1H, J = 15.5 Hz, COCH = CH), 7.01 (d, 1H, J = 2.0 Hz, Ar-H), 6.99 (s, 1H, H-3), 5.58 (d, 1H, J = 3.0 Hz, H-2'), 5.34 (dd, 1H, J = 2.5, 4.5 Hz, H-3'), 5.29 (brs, 2H, PhCH₂), 4.91 (d, 1H, J = 4.0 Hz, H-1'), 4.32 (dd, 1H, J = 5.0, 10.5 Hz, H-4'_a), 4.18 (dd, 1H, J = 2.0, 12.5 Hz, H-4'_b), 4.05 (s, 3H, OCH₃), 2.72 (s, 3H, CH₃), 2.23, 2.20 (2 s, 6H, CH₃CO); ¹³C NMR (125 MHz, CDCl₃): δ 185.2 (C=O),



170.0, 169.5 (CH₃CO), 159.5 (C-4), 152.0 (C-1), 148.3, 147.1 (Ar-C), 143.3 (COCH = CH), 136.8, 128.6, 128.0, 127.6, 127.3 (Ar-C), 123.4 (COCH = CH), 122.5 (C-2), 121.6, 113.2, 111.5 (Ar-C), 109.0 (C-3), 79.7 (C-1'), 78.3 (C-2'), 77.9 (C-3'), 72.0 (C-4'), 71.2 (PhCH₂), 55.9 (OCH₃), 14.6 (CH₃); ESI-MS: 557.1 [M + Na]⁺; anal. calcd for C₃₀H₃₀O₉ (534.55): C, 67.41; H, 5.66%; found: C, 67.31; H, 5.70%.

Compound 31. Yellow solid; *R*_f: 0.18 (hexane–EtOAc; 1 : 3); mp 99–101 °C [EtOH]; [α]_D²⁵ +13 (*c* 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 7.70 (d, 1H, *J* = 15.5 Hz, COCH = CH), 7.56 (d, 1H, *J* = 7.5 Hz, Ar-H), 7.49 (t, 2H, *J* = 8.0, 7.5 Hz, Ar-H), 7.41 (d, 1H, *J* = 7.5 Hz, Ar-H), 7.27 (d, 1H, *J* = 8.0 Hz, Ar-H), 7.22 (d, 1H, *J* = 2.0 Hz, Ar-H), 7.03 (d, 1H, *J* = 15.5 Hz, COCH = CH), 6.97 (d, 1H, *J* = 8.5 Hz, Ar-H), 6.80 (s, 1H, H-3), 5.27 (brs, 2H, PhCH₂), 4.83 (d, 1H, *J* = 5.5 Hz, H-1'), 4.12 (m, 1H, H-2'), 4.02 (s, 3H, OCH₃), 3.88 (dd, 1H, *J* = 3.0, 12.5 Hz, H-3'_a), 3.76 (dd, 1H, *J* = 5.5, 12.5 Hz, H-3'_b), 2.69 (s, 3H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 185.7 (C=O), 158.9 (C-4), 152.1 (C-1), 151.3, 148.3 (Ar-C), 143.5 (COCH = CH), 136.7, 128.6, 128.0, 127.5, 127.3 (Ar-C), 123.5 (COCH = CH), 122.0 (C-2), 121.5, 113.1, 111.5 (Ar-C), 108.1 (C-3), 72.9 (C-1'), 71.2 (PhCH₂), 68.3 (C-2'), 63.5 (C-3'), 55.9 (OCH₃), 14.5 (CH₃); ESI-MS: 461.1 [M + Na]⁺; anal. calcd for C₂₅H₂₆O₇ (438.47): C, 68.48; H, 5.98%; found: C, 68.30; H, 6.13%.

Compound 32. Yellow solid; *R*_f: 0.15 (hexane–EtOAc; 1 : 3); mp 94–96 °C [EtOH]; [α]_D²⁵ +7 (*c* 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 8.44 (s, 1H, Ar-H), 8.22 (d, 1H, *J* = 8.0 Hz, Ar-H), 7.85 (d, 1H, *J* = 8.5 Hz, Ar-H), 7.73 (d, 1H, *J* = 15.5 Hz, COCH = CH), 7.58 (t, 1H, *J* = 8.0, 8.5 Hz, Ar-H), 7.26 (d, 1H, *J* = 15.5 Hz, COCH = CH), 6.74 (s, 1H, H-3), 4.72 (d, 1H, *J* = 5.0 Hz, H-1'), 3.99–3.79 (m, 1H, H-2'), 3.88 (dd, 1H, *J* = 3.0, 12.5 Hz, H-3'_a), 3.76 (dd, 1H, *J* = 5.5, 12.5 Hz, H-3'_b), 2.68 (s, 3H, CH₃); ¹³C NMR (125 MHz, CDCl₃): δ 185.6 (C=O), 158.7 (C-4), 149.8 (C-1), 139.9 (COCH = CH), 135.5, 133.6, 129.6, 126.3, 124.2 (Ar-C), 123.5 (COCH = CH), 122.1 (C-2), 107.5 (C-3), 72.8 (C-1'), 68.4 (C-2'), 63.3 (C-3'), 14.4 (CH₃); ESI-MS: 370.26 [M + Na]⁺; anal. calcd for C₁₇H₁₇NO₇ (347.32): C, 58.79; H, 4.93%; found: C, 58.62; H, 5.10%.

Compound 33. Yellow oil; *R*_f: 0.38 (hexane–EtOAc; 1 : 1); [α]_D²⁵ +12 (*c* 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 7.73 (d, 1H, *J* = 15.5 Hz, COCH = CH), 7.59 (d, 2H, *J* = 7.0 Hz, Ar-H), 7.52 (t, 2H, *J* = 7.5 Hz each, Ar-H), 7.45 (d, 1H, *J* = 7.0 Hz, Ar-H), 7.30 (dd, 1H, *J* = 1.5, 8.5 Hz, Ar-H), 7.25 (d, 1H, *J* = 2.0 Hz, Ar-H), 7.02 (d, 1H, *J* = 15.5 Hz, COCH = CH), 7.01 (d, 1H, *J* = 8.5 Hz, Ar-H), 6.87 (s, 1H, H-3), 6.15 (d, 1H, *J* = 8.0 Hz, H-1'), 5.70–5.68 (m, 1H, H-2'), 5.31 (brs, 2H, PhCH₂), 4.48 (dd, 1H, *J* = 8.5, 12.5 Hz, H-3'_a), 4.05–4.02 (m, 4H, OCH₃, H-3'_b), 2.74 (s, 3H, CH₃), 2.2, 2.18 (2 s, 6H, CH₃CO); ¹³C NMR (125 MHz, CDCl₃): δ 184.9 (C=O), 170.1, 169.7, 169.4 (3C, CH₃CO), 159.9 (C-4), 152.1 (C-1), 148.4, 146.3 (Ar-C), 143.5 (COCH = CH), 136.8, 128.6, 128.0, 127.3 (Ar-C), 123.5 (COCH = CH), 122.5 (C-2), 121.4, 113.0, 111.5 (Ar-C), 110.6 (C-3), 71.2 (PhCH₂), 70.2 (C-1'), 66.4 (C-2'), 62.0 (C-3'), 55.9 (OCH₃), 20.8, 20.7, 20.6 (3C, CH₃CO), 14.5 (CH₃); ESI-MS: 587.2 [M + Na]⁺; anal. calcd for C₃₁H₃₂O₁₀ (564.58): C, 65.95; H, 5.71%; found: C, 65.86; H, 5.89%.

Compound 34. Yellow oil; *R*_f: 0.44 (hexane–EtOAc; 1 : 1); [α]_D²⁵ +11 (*c* 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ 8.48 (s, 1H, Ar-H), 8.26 (d, 1H, *J* = 8.0 Hz, Ar-H), 7.89 (d, 1H, *J* = 7.5 Hz, Ar-

H), 7.75 (d, 1H, *J* = 15.5 Hz, COCH = CH), 7.62 (t, 1H, *J* = 8.0 Hz each, Ar-H), 7.28 (d, 1H, *J* = 15.5 Hz, COCH = CH), 6.85 (s, 1H, H-3), 6.02 (d, 1H, *J* = 3.5 Hz, H-1'), 5.53–5.50 (m, 1H, H-2'), 4.43 (dd, 1H, *J* = 3.0, 12.5 Hz, H-3'_a), 3.94 (dd, 1H, *J* = 5.5, 12.0 Hz, H-3'_b), 2.68 (s, 3H, CH₃), 2.12, 2.11, 2.09 (3 s, 9H, CH₃CO); ¹³C NMR (125 MHz, CDCl₃): δ 183.8 (C=O), 170.0, 169.8, 169.1 (3C, CH₃CO), 160.6 (C-4), 148.7 (C-1), 140.3 (COCH = CH), 136.4, 133.9, 129.7, 127.9, 126.0, 125.5 (Ar-C), 122.2 (COCH = CH), 122.8 (C-2), 110.5 (C-3), 70.1 (C-1'), 66.1 (C-2'), 61.6 (C-3'), 20.7, 20.6, 20.5 (3C, CH₃CO), 14.6 (CH₃); ESI-MS: 496.1 [M + Na]⁺; anal. calcd for C₂₃H₂₃NO₁₀ (473.43): C, 58.35; H, 4.90%; found: C, 58.47; H, 5.10%.

MTT cell proliferation assay

The anti-proliferative effects of the *C*-glycosyl-3-cinnamoylfuran derivatives (7–34) were assessed by a MTT proliferation assay.²⁶ For this experiment, cells were seeded in triplicate in 96 well plates and incubated overnight. Post incubation cells were treated with compounds at various concentrations (5 μM, 10 μM, 20 μM and 50 μM) for 48 h. After the treatment, 200 μl of MTT solution (0.5 mg ml⁻¹) were added to each well and incubated at 37 °C for 3 h. Subsequently, the MTT media was discarded and purple colored formazan crystals were dissolved using DMSO. The absorbance was measured at 570 nm in a 96 well micro-plate reader (Thermo, Multiskan Go).

Clonogenic survival assay

In 6 well plates around 200 MCF-cells were seeded in each of the wells and cells were allowed to attach to the plates for 2 h. Then the cells were treated with different concentrations of compound 24 (0 nM, 100 nM, 500 nM, 1.25 μM, 2.5 μM and 5 μM) for the next 15 days with continuous change of media on every 3rd day with or without compounds. After 15 days the cells were washed with 1X PBS and incubated with 3 ml of a mixture of 3.7% formaldehyde in 1X PBS and 0.5% crystal violet for 30 minutes. The formaldehyde crystal violet mixture was carefully removed by rinsing with tap water and plates were allowed to dry at room temperature. Finally, the image was captured using Gel Doc XR + (Bio-Rad) and colony numbers were counted.

Cell cycle arrest assay by propidium iodide staining

MCF-7 cells were treated with increasing concentrations (0, 5 μM, 10 μM, 20 μM, 30 μM) of compound 24 for 24 h and 2 μM of nocodazole was used as a positive control for G2 arrest. Post treatment cells were converted into single cell suspension and fixed with 75% ethanol overnight at –20 °C. Cells were centrifuged and resuspended in 1X PBS for 2 h followed by RNase A (20 μM) treatment for 2 h at 37 °C. Finally, propidium iodide was added and incubated for 20 minutes at room temperature. Flow cytometric analysis was immediately performed using a FACS Verse instrument (BD).

Analysis of apoptosis by annexin V-FITC/PI

To determine the apoptosis-inducing ability of compound 24 on MCF-7 cells, a double staining method with annexin V-FITC and



PI was performed using an assay kit (BD bioscience kit). MCF-7 cells were seeded in a 6 well plate and incubated overnight followed by treatment with various concentrations of compound **24** (5 μ M, 10 μ M, 20 μ M and 30 μ M) for 48 h. After treatment, the cells were washed with 1X PBS and resuspended in 100 μ l of binding buffer. Finally, cells were incubated with 5 μ l of annexin V-FITC and 5 μ l of PI for 15 min at room temperature in the dark and flow cytometric analysis was performed using a BD Verse FACS machine.

Proteome Profiler array

A Human Apoptosis Array Kit from R&D Systems (USA) was used for determining the apoptosis-related proteins following the manufacturer's protocol. Briefly, the cells were treated with compound **24** at 30 μ M concentration for 48 h and a DMSO control was kept. 250 μ g protein from each sample was incubated overnight with the array. Finally, the data was measured by developing the membrane and then the pixel density was calculated using Image J software. Data are representative of two independent experiments and the bar graph shows mean \pm SEM (* p < 0.05, ** p < 0.01, *** p < 0.001, ns = not significant).

Caspase 3 activity assay

A colorimetric assay following the manufacturer's protocol (Caspase 3 Assay Kit (Colorimetric) (ab39401)) was used to measure the caspase activity. MCF-7 cells were treated with compound **24** at a concentration of 30 μ M for 24 h and 48 h. The RIPA buffer method was used to prepare the cell lysates. Each cell lysate was incubated with 50 μ l of 2 \times reaction buffer (containing 10 mM DTT) and 5 μ l of the 4 mM DEVD-p-NA substrate (200 μ M) and kept at 37 $^{\circ}$ C for 2 h. The OD was measured at 400 nm in a micro-plate reader and then the graph was plotted.

Quantitative structure activity relationship (QSAR) analysis

Based on the experimental results, the molecular structures were compared with the IC₅₀ value to elucidate a quantitative structure-activity relationship. The 3D-QSAR module of the Schrödinger program was used to derive the atom-based analysis. All molecular structures were prepared and minimized using Maestro GUI. The models were processed by categorizing all 28 molecules into a training set and a test set. The model divides the space of grids into uniform-sized cubes that reflect the biological relationship using structural features, corresponding through positive (blue) and negative (red) contours. Characterization of molecules is performed using independent variables for structural components to reflect statistically significant results. Model building was performed using partial least-squares regression (PLS). Detailed methodology and principles are similar to the reports published earlier.³⁹

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