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Synthesis of neolignans as microtubule stabilisers

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ABSTRACT

Tubulin is a well established target for anticancer drug development. Lignans and neolignans were synthesized as tubulin interacting agents. Neolignans **10** and **19** exhibited significant anticancer activity against MCF-7 and MDAMB-231 human breast cancer cell lines. Both the compounds effectively induced stabilization of microtubule at 4 and 20 μM concentrations respectively. Neolignan **10** induced G2/M phase arrest in MCF-7 cells. Docking experiments raveled that **10** and **19** occupied the same binding pocket of paclitaxel with some difference in active site amino acids and good bioavailability of both the compounds. In in vivo acute oral toxicity **10** was well tolerated up to 300 mg/kg dose in Swiss-albino mice.

1. Introduction

Lignans and neolignans are important biodynamic agents with varied structural diversity. In plants, these are produced as secondary metabolites derived from phenylpropanoids (C6–C3). Both lignans and neolignans are dimeric compounds with different linkages. Lignans are formed by C2–C2′ linkage through carbons of propyl chains, while other C–C linkages are known as neolignans. Both lignans and neolignans are widespread in plants.[1–4] Lignans and neolignans have exhibited a wide range of biological activities such as antimalarial,[5,6] antitubercular,[7,8] anticancer,[9–14] apoptosis inducers,[15,16] antiviral[17,18] and antioxidants,[19] etc. More interestingly, lignans and neolignans have been good ligands for estrogen receptors,[19] aldose reductase,[20,21] tyrosinase,[22] topoisomerase II,[23] $GABA_A$ receptor,[24] voltage gated K^+ channels,[25] etc. acting as inhibitors by curtailing these enzymatic actions.

Several potent leads like podophyllotoxin (PDT) as anticancer,[26] silymarin, phyllanthin, hypophyllanthin and cleomiscosins as hepatoprotectives[27] have been obtained from this class of compounds. Podophyllotoxin is an aryl tetralin lignan isolated from *Podophyllum* spp.[26] It acts as a mitotic inhibitor by binding reversibly to tubulin and inhibiting microtubule assembly.[26,28] Due to toxicity reasons podophyllotoxin could not be developed as anticancer drug. However, two of its semisynthetic derivatives, etoposide and teniposide are used clinically to treat small-cell lung cancer, testicular cancer, leukemia, lymphoma, and other cancers.[26]

3,4,5-Trimethoxyphenyl unit in several antitubulins plays a crucial role in interacting with tubulin. There are antitubulin agents like podophyllotoxin, colchicine, combretastatin A4, etc. possessing this unit.[26] We designed lignans (prototype-I) and neolignans (prototype-II) as possible anticancer agents with this fragment (Fig. 1). Several analogues were synthesized and evaluated against human cancer cell lines. The mode of action of active compounds was evaluated against tubulin polymerase enzyme. The tubulin interaction was further confirmed by in silico docking studies. The most active compound **10** was evaluated for its effect on cell cycle phases. It was also evaluated for acute oral toxicity in Swiss-albino mice at various doses.

2. Results

2.1. Chemistry

The synthetic strategy was as depicted in Scheme 1. Firstly, 3,4,5-trimethoxycinnamic acid (**1**) was esterified to corresponding ethyl ester (**2**) by treating it with thionyl chloride and ethanol at room temperature. The *p*-methoxy group of ester **2** was selectively demethylated by treatment with anhydrous aluminium chloride in

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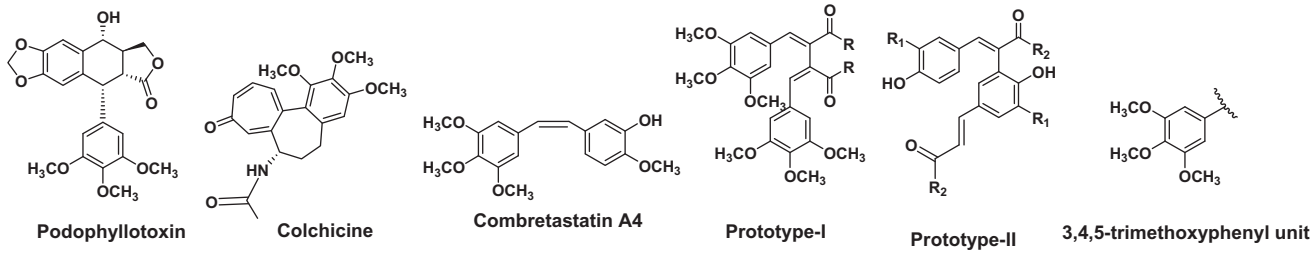
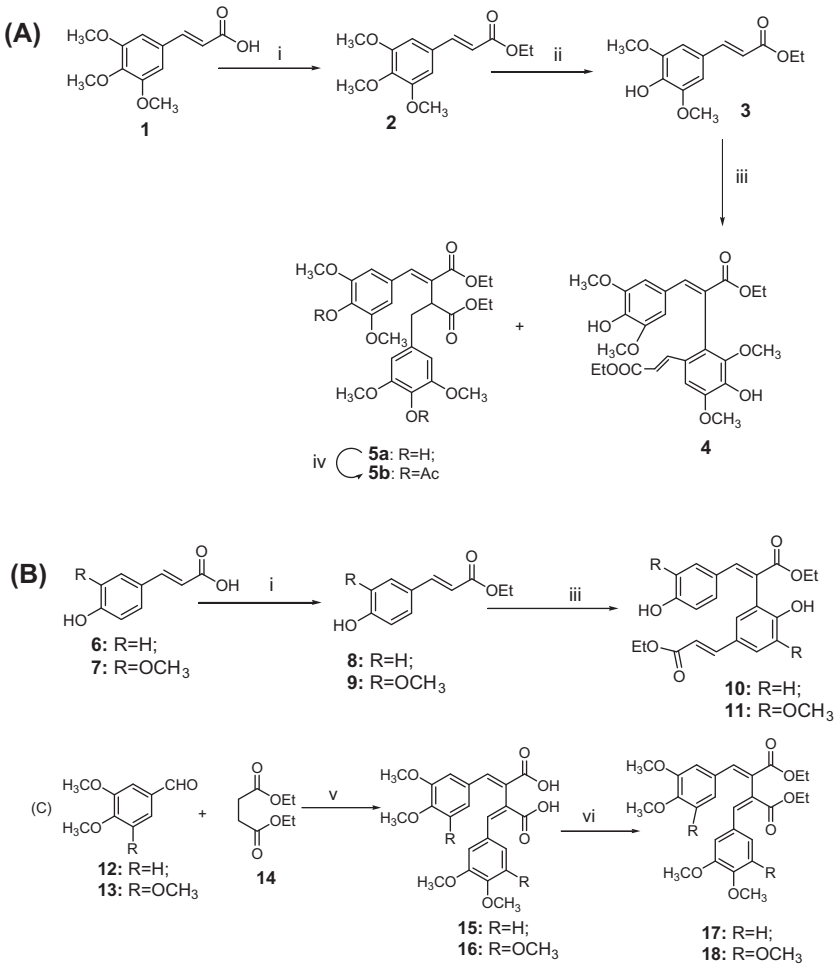


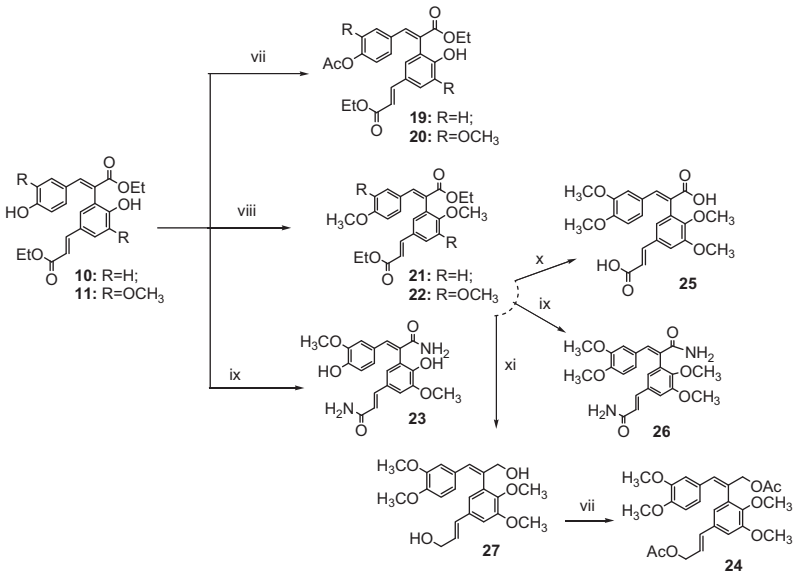
Figure 1. Structures of antitubulins (podophyllotoxin, colchicine, CA4), prototypes I and II, and trimethoxyphenyl fragment.



Scheme 1. Reagents and conditions: (i) EtOH, $SOCl_2$, RT, 2 h, 89–94%; OR concd H_2SO_4, EtOH, 85 °C, 4 h, 84–91%; (ii) anhydrous $AlCl_3$, DCM, 40 °C, 2 h, 84%; (iii) aqueous KOH, $K_3[Fe(CN)_6]$, benzene, RT, 30 min, neolignan 21%, lignan 24–29%; (iv) dry pyridine, Ac_2O, RT, overnight, 92%; (v) NaH, DMF, reflux, 4 h, 43–49%; (vi) Et_2SO_4, K_2CO_3, acetone/DMSO = 3:1, reflux, 2 h, 82%.

dry methylenechloride to get 3,5-dimethoxy, 4-hydroxycinnamic acid ethyl ester (**3**).[29] The free radical coupling of ester **3** was done in potassium ferricyanide solution in aqueous alkali-benzene system. Use of phase transfer catalyst (tetrabutyl ammoniumbromide, TBAB) was not beneficial in this reaction, as reaction failed in presence of it. In case of ester **3**, two products were formed. Compound **4** was identified as neolignan and **5a** was identified as lignan. Similarly ethyl esters of **8** and **9** were processed to get neolignans **10** and **11** respectively. In case of **8** and **9**, we did not get lignans, only neolignans were obtained. But, the yields in these reactions were poor (21–29% only). So, alternatively, we synthesized these lignans through Stobbe's condensation. Here, substituted aromatic aldehyde (**12** or **13**) was condensed with diethyl succinate (**14**) in presence of sodium hydride in dry dimethylformamide (DMF) under reflux condition in inert atmosphere (N_2). The yields of the lignans **15** and **16** as diacids were significantly higher (43–49%) by this method. The diacids were further converted to ethyl esters (**17** and **18**) by using diethyl sulfate in anhydrous potassium carbonate in dry acetone.

Further these neolignans **10** and **11** were modified to various simple derivatives (Scheme 2) by acetylation (**19** and **20**), methylation (**21** and **22**) and amide formation (**23** and **26**), etc. using standard protocols.[30] Ester **22** was hydrolysed to corresponding diacid (**25**) using 5% methanolic–KOH at 60 °C. Ester **22** was treated with 30% aqueous ammonia at 0 °C to get corresponding diamide (**26**). On reduction with lithium borohydride in THF ester **22** yielded a



Scheme 2. Reagents and conditions: (vii) pyridine, Ac_2O, RT, overnight, 89–92%; (viii) Me_2SO_4, K_2CO_3, DMF, reflux, 80 °C, 2 h, 87–91%; (ix) 30% aqueous NH_3, 0 °C–rt, 2 h, 58–62%; (x) 5% methanolic–KOH, 60 °C, 2 h, 86%; (xi) $LiBH_4$, THF, 0 °C, 4 h, 87%.

diol derivative **27**. Diol **27** was finally acetylated with pyridine–Ac_2O to get diacetyl derivative **24**. All the compounds were characterized by spectroscopy.

2.2. Biological results

All the lignans and neolignans were evaluated against MCF-7 and MDAMB-231 human breast cancer cell lines (Table 1). Only eight of the derivatives showed some cytotoxicity against breast cancer cell lines. Rests of the derivatives were inactive at 100 μM. The best compound of the series was **10** exhibiting IC_{50} 12 and 15 μM against MCF-7 and MDAMB-231, respectively. However, IC_{50} of **10** and **19** were much better when incubation time was enhanced to 48 and 72 h.

2.3. Effect of lead molecule on cell cycle phases of MCF-7 and MDA-MB-231 cells

MCF-7 cells were treated with compound **10** for 24 h and cell cycle phase distribution was recorded with PI-staining method (Fig. 2). Neolignan **10** showed significant increase in G2 phase at all concentrations evaluated suggesting G2/M arrest, along with a dose dependent increase in sub-diploid population indicating possible apoptosis. There was also decrease in S-phase population for all the concentrations of compound **10** in comparison to untreated control. However, G1 phase was not affected by any of the concentrations of compound **10**.

In case of MDA-MB-231 cells, compound **10** did not affect cell division cycle in treated cells, except dose-dependent increase of

Table 1
In vitro cytotoxicity of lignans and neolignans by MTT assay

Compound no.	Mol wt.	MCF-7 IC_{50}[#] (μM)	MDAMB-231 IC_{50}[#] (μM)	HEK-293 IC_{50}[#] (μM)
4	502	74 ± 0.012	92 ± 0.026	Nd
5a	502	—[*]	82 ± 0.037	Nd
5b	586	—	—	Nd
10	382	12 ± 0.0052 (24 h) 9.29 ± 0.03 (48 h) 3.27 ± 0.02 (72 h)	15 ± 0.0085 (24 h) 6.47 ± 0.03 (48 h) 0.45 ± 0.02 (72 h)	12 μM ± 0.12
11	442	—	—	Nd
17	470	Nd	Nd	Nd
18	530	100 ± 0.0013	—	Nd
19	424	66 ± 0.003 (24 h) 9.65 ± 0.02 (48 h) 0.82 ± 0.06 (72 h)	16 ± 0.0025 (24 h) 3.22 ± 0.03 (48 h) 0.21 ± 0.05 (72 h)	20 μM ± 0.233
20	484	—	—	Nd
21	410	—	—	Nd
22	470	—	—	Nd
23	384	100 ± 0.0019	80 ± 0.099	Nd
24	470	—	—	Nd
25	414	Nd	85 ± 0.022	Nd
26	412	100 ± 0.018	32 ± 0.0068	Nd
27	386	—	—	Nd
28	470	100 ± 0.011	100 ± 0.0247	Nd
Podophyllotoxin	414	64.99 ± 4.38	35.7 ± 11.80	50 μM ± 0.224
Tamoxifen	371	9 ± 0.003	10 ± 0.010	26 μM ± 0.22

[*] Means not active, IC_{50} >100 μM, Nd = not done.
[#] Incubation time = 24 h.

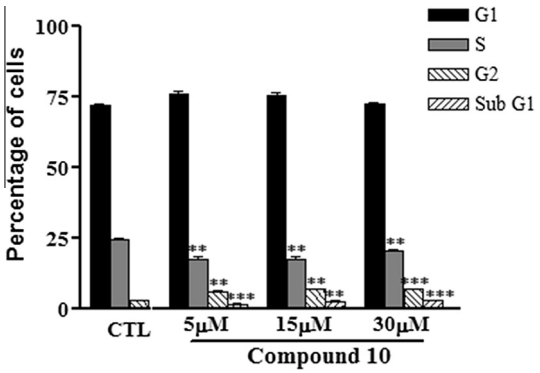


Figure 2. Effect of neolignan **10** in cell cycle phases of MCF-7 cells.

sub-diploid cells at higher concentrations (15 and 30 μM). Compound **10** might have only caused apoptosis in MDA-MB-231 without causing cell cycle arrest at any phase. This also suggested that compound **10** affects cell cycle phases of MDA-MB-231 differently than MCF-7 cells (Fig. 3).

2.4. Biochemical measure of tubulin polymerization activity of lead molecules

Neolignans **10** and **19** were further evaluated for their effect on tubulin polymerisation (Fig. 4). For this experiment, we incorporated tubulin destabilizing agent PDT and stabilizing agent taxol as controls to improve reliability of our assay. As can be seen in Figure 4, both the neolignans showed stabilization of tubulin assembly similar to taxol at various concentrations (10, 20 and 40 μM) whereas, the standard tubulin destabilizing agent PDT, effectively inhibited tubulin polymerization in comparison to control groups. Compounds **10** showed better stabilization of microtubule polymerization in comparison to compound **19**.

2.5. Effect of lead molecule on actin–tubulin cytoskeleton structure with confocal microscopy

In order to observe the phenotypic effect of compound **10** (as showed better activity in tubulin polymerization assay) on cellular cytoskeletal network of actin and tubulin, MCF-7 cells were immunostained and analyzed under confocal microscope. As illustrated in Figure 5, substantial stabilization of microtubules in the form of bundle like appearance was observed in paclitaxel-treated cells that were used as positive control in this assay. However, in compound **10** treated cells, stabilization of tubulin network was not apparent up to the level in comparison to positive control

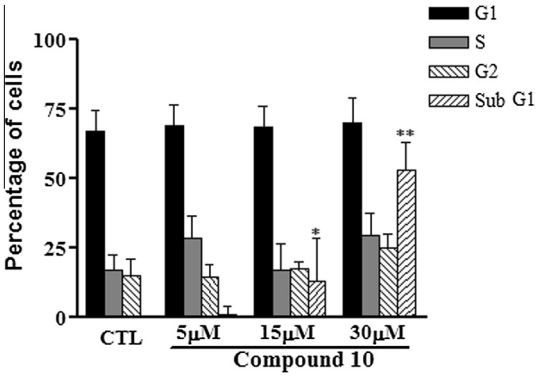


Figure 3. Effect of compound **10** in cell cycle phases of MDA-MB-231 cells.

groups. No changes in actin network were evident in control as well as in treatment groups.

2.6. Molecular docking for binding studies

The modulation of anticancer activity of **10** and **19** was elucidated through interaction with tubulin polymerase and identifying binding site pocket. The molecular docking results also confirmed that both **10** and **19** stabilize the polymerisation of tubulin. The orientations and binding affinities (in terms of total score) of **10** and **19** were established towards tubulin (PDB ID: 1TUB). The docking reliability was validated by using the known X-ray crystal structure of tubulin complexed with taxol and docked conformation with the highest total score of 6.3796 was selected as the most probable binding conformation. The low root mean-square deviation (RMSD) of 0.6014 Å between the docked and the crystal conformations indicates the high reliability of Surflex-dock software in reproducing the experimentally observed binding mode for taxol. As shown in Figure 6A, redocked molecules were almost in the same position with co-crystallized at the active site. Crystallography data showed that the amino acid Threonine-276 is the 'gatekeeper' residue, an important determinant of stabilizing specificity in the tubulin binding pocket.

The docking results of **19** against target protein showed high binding affinity docking score indicated by total score of 8.6059 and formation of two hydrogen bonds of length 2.0 and 1.8 Å through hydrophobic residues THR-276 and HIS-229. In docking pose, the conserved binding site pocket amino acid residues within a selection radius of 4 Å from bound ligand were hydrophobic residue Val-23 (Valine) PHE-272; nucleophilic (polar, hydrophobic), for example, THR-276 (Threonine), SER-232, SER-236, SER-277 (Serine); basic LYS-218 (Lysine), ARG-320 (Arginine) CYS-213 (Cysteine), GLN-281(Glutamine), HIS-299 (Histidine); acidic (polar, negative charged), for example, ASP-26 (Aspartic acid), Hydrophobic, for example, ALA-233, ARG-278 (Alanine), LEU-217, LEU-219, LEU-230, LEU-275, LEU-371 (Leucine), and imino acids PRO-274, PRO-360 (Proline), as a result, the bound compound **19** showed strong hydrophobic interactions with tubulin, thus leading to more stability and activity in this compound (Fig. 6B).

The binding affinity obtained in the docking study allowed a comparison between the activities of the **10** to be compared to that of the standard anticancer drug taxol. Compound **10** showed a higher binding affinity against tubulin, the target protein. During the comparison of the nature of interaction between the binding pocket amino acid residues of target protein and compound **10**, it was found that the compound **10** showed molecular interactions with conserved hydrophobic amino acid residues, thus leading to more stability and potency (Table 2). The docking results for **10** showed that the compound docked on tubulin with a high binding affinity docking score indicated by its total score of 6.3796 and also showed the formation of a two H-bond of length 1.9 and 1.8 Å to the acidic residues, Glu-22 and basic (hydrophobic) residues, Lys-19. The **10** tubulin-docked complex also showed a similar type of binding site residues within a radius of 4 Å of bound ligand such as nucleophilic (polar, hydrophobic), for example, SER-232, SER-236 (Serine); basic LYS-19 (Lysine), ARG-320, HIS-299 (Histidine); acidic (polar, negative charged), for example, ASP-26 (Aspartic acid); Hydrophobic, for example, ALA-233 (Alanine), ARG-278, ARG-369 (Arginine) LEU-219, LEU-230, LEU-275, LEU-371 (Leucine), Val-23 (Valine), PHE-272 (Phenylalanine); imino acids PRO-274, PRO-360 (Proline); and acidic (polar, negative charged) residues, for example, GLU-22 (Glutamic acid), therefore, the docked molecule also showed a strong hydrophobic interaction with tubulin, thus leading to more stability (Fig. 6C).

The docking results for the negative control compound podophyllotoxin (tubulin inhibitor) with tubulin showed a low

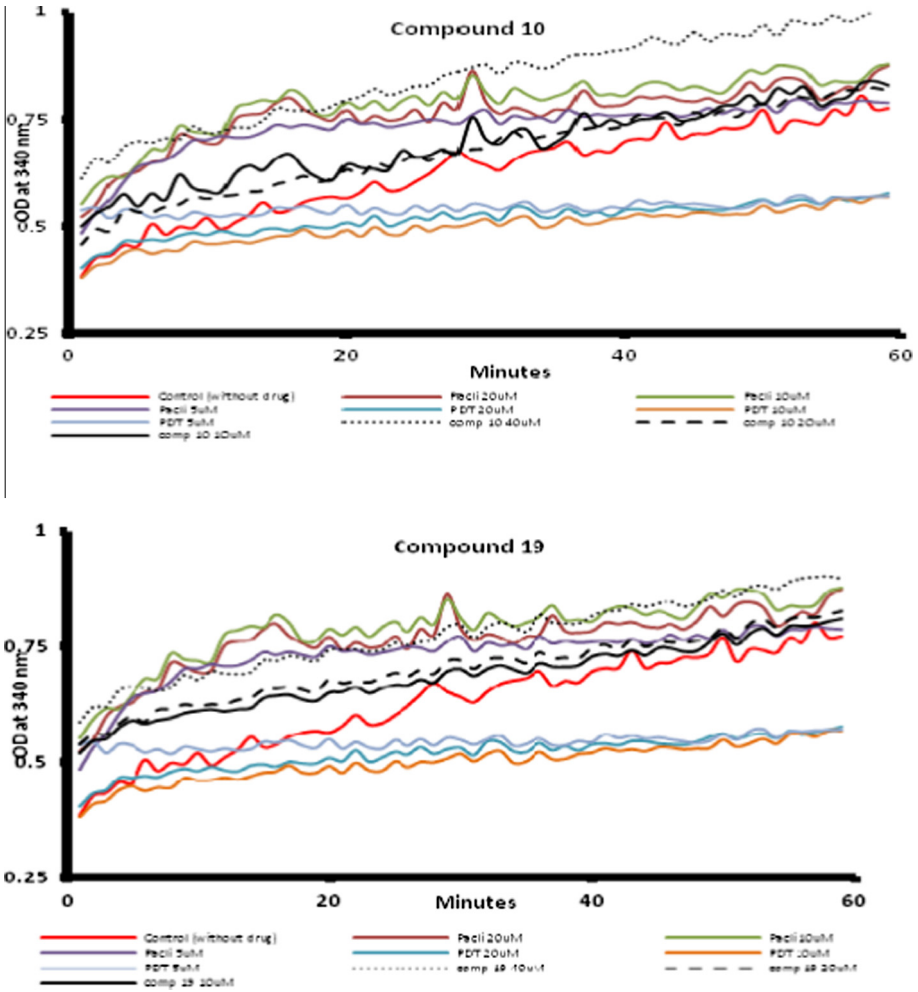


Figure 4. Kinetics of tubulin polymerisation by compounds **10** and **19** at different concentrations with controls.

binding affinity, docking score indicated by a low total score of 2.9284 without any H-bond (hydrogen bond) formation (Fig. 6D), in comparison to the docking score of anticancer known stabilizing agent taxol, which showed a total score of 6.3796 (Table 2). Thus, the docking procedure of Surflex-dock software (Sybyl-X 1.3) in reproducing the experimental binding affinity seems reliable, and therefore predicted as true positive.

2.7. In silico ADMET analysis

Neolignans **10** and **19** were further evaluated for several physiochemical properties (ADME) related to pharmacokinetics. The logP value indicates about hydrophilicity and lipophilicity of the molecule which has an important role for the transportation of the compound in the body. Both the compounds follow Lipinski's rule of five for good bioavailability. Both possess molecular weights less than 500 (mw <500 kDa), number of hydrogen bond donors less than 5 (H_d <5), H-bond acceptors less than 10 (H_a <10) and Log*P* value less than 5 (Log*P* <5). Low hydrophilicity and therefore a high Log*P* value may lead to poor absorption or permeation. Both the compounds showed calculated Log*P* values less than 5, so these compounds will have good hydrophilicity with moderate lipophilicity and hence should be able to gain access to membrane surfaces. Log*P* value has also been linked to blood–brain barrier penetration and utilized to predict cell membrane permeability. The process of excretion, which eliminates the compound from the human body, depends on its molecular weight and Log*P*.[31] Molecules with intermediate lipophilicities have a better chance of arriving at the receptor site.[32] Typically, low solubility is associated with poor absorption, so the general aim is to avoid poorly soluble compounds. The aqueous solubility (Log*S*) of a compound significantly affects its absorption and distribution characteristics. The calculated Log*S* values of **10** and **19** were within the acceptable range. Other calculations related to solubility, serum protein binding, blood–brain barrier (Log BB and apparent MDCK cell permeability), gut–blood barrier (Caco-2 cell permeability), predicted central nervous system activity, number of likely metabolic reactions, hERG K^+ ($Log IC_{50}$) channel blockage, skin permeability (Kp), and human oral absorption in the gastrointestinal tract showed these values for both **10** and **19** within the standard ranges for good bioavailable drugs (Table 3).

2.8. In vivo acute oral toxicity of neolignan 10

No observational changes, morbidity and mortality were observed throughout the experimental period up to the dose level of 1000 mg/kg body weight. No morbidity or any other gross observational changes could be noticed in the any group of animals treated with compound **10**. Blood and serum samples upon analysis showed non-significant changes in all the parameters studied like total haemoglobin level, RBC count, WBC count, differential leucocytes count, SGPT, ALKP, creatinine, triglycerides, cholesterol, albumin, serum protein (Table 4 and Fig. 7). However, SGOT showed significant increase in group of animals treated with the test drug

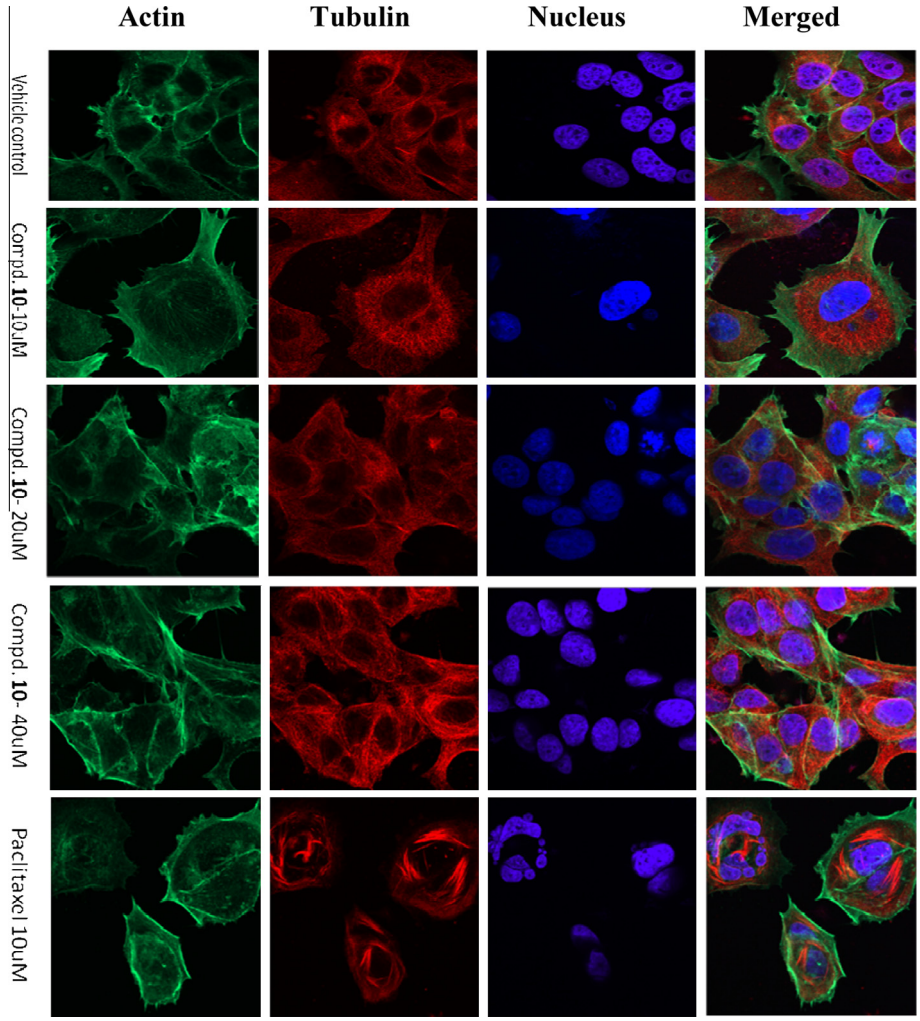


Figure 5. Effect of compound **10** on actin–tubulin cytoskeleton structure in MCF-7 cell line with confocal microscopy.

at 1000 mg/kg body weight as compare to control groups. Animals on gross pathological study showed no changes in any of the organs studied including their absolute and relative weight (Fig. 8). Therefore, the experiment showed that compound **10** is well tolerated by the Swiss albino mice up to the dose level of 300 mg/kg body weight as a single acute oral dose. However, sub-acute and or chronic experiment with the test drug needs to be carried out to look for any adverse effect on repeated exposure to compound **10** for its future development.[33]

3. Discussion

Microtubules are a component of cytoskeleton, involved in maintaining cell structure and cell division including the formation of spindles. These are polymers of α- and β-tubulin dimers. The polymerisation of tubulins to microtubulins is a reversible process which is in a dynamic equilibrium. A number of anticancer molecules bind to tubulin and modify its activation state. They can either stabilize the polymerisation process or destabilize (inhibit) it. In both the cases the microtubule dynamics is disturbed, which leads to cell cycle arrest and can lead to apoptosis. Owing to their essential role in forming dynamic spindle apparatus in mitosis, microtubules are considered an ideal target for anticancer drug development.[34] Taxol and epothilones block dynamic instability by stabilizing GDP-bound tubulin in the microtubule. On the contrary, podophyllotoxin, vincristine, vinblastine, and colchicine block the polymerisation of tubulins to microtubulin. Both types of tubulin interacting agents have different binding sites in the tubulin polymerase.

The lignans and neolignans synthesized were supposed to inhibit tubulin polymerase enzyme similar to podophyllotoxin. But, unexpectedly, these compounds stabilized the tubulin polymerisation process very similar to taxol. Docking experiments showed significant binding affinities of **10** and **19** to tubulin, but both the compounds possessed moderate cytotoxicities in in vitro experiments against human breast cancer cell lines (MCF-7 and MDAMB-231). It might be because the amino acids involved in hydrogen bonding with **10** and **19** are different from taxol. **19** has only one amino acid (THR-276) common to taxol while, **10** has none. These bindings might be responsible for eliciting cytotoxicity in the molecule. Due to this, although **10** and **19** possessed tubulin stabilization property (similar to taxol) with better binding affinities, these could not induce better cytotoxicity against human cancer cell lines. Anti-tubulin agents can act differently due to their differential binding sites or other indirect mechanism leading to modulation of microtubule dynamics. While some anti-tubulin agents act by promoting polymerization (taxanes) or by augmenting depolymerisation (vincas) of microtubules, there are some more class of microtubule inhibitors, like EM011, which do not cause condensation of tubulin by altering total microtubule mass. EM011, a synthetic noscapine derivative, is a well established microtubule interfering agent which binds tubulin with high

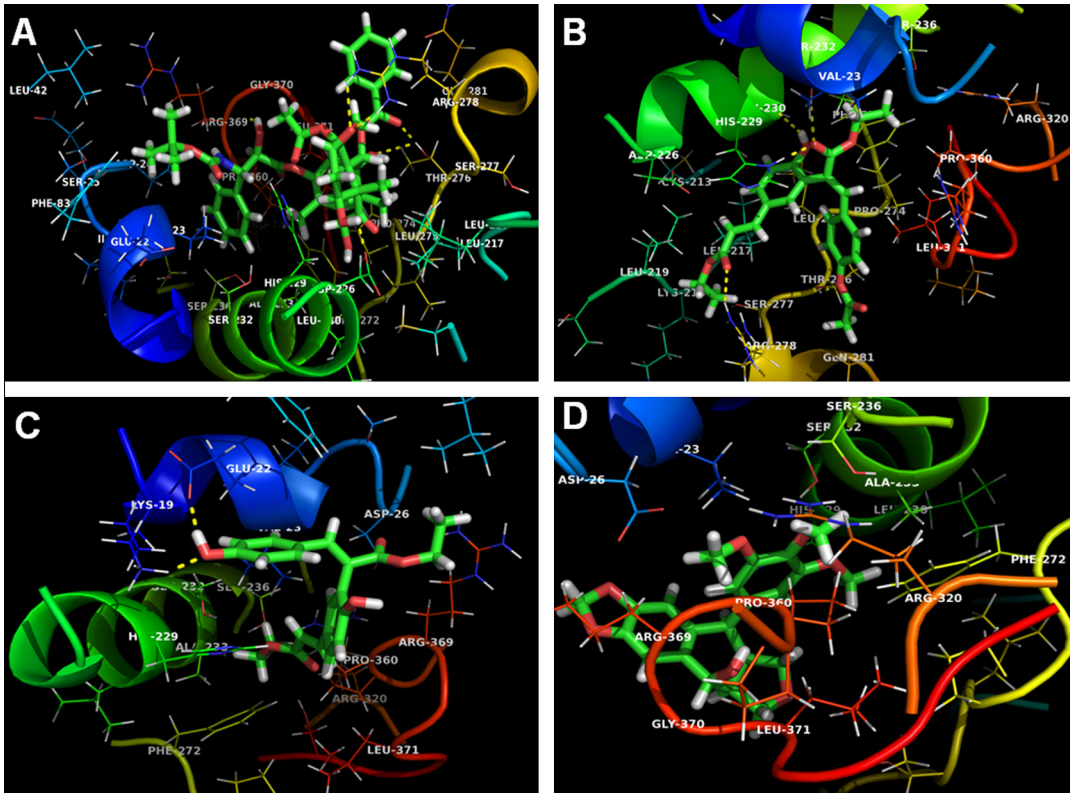


Figure 6. In silico molecular docking studies elucidating the possible mechanisms of **19** and **10** induced modulation of tubulin protein. The docking studies were carried out using SYBYL-X 1.3, Tripos International. (A) The co-crystallized taxol was redocked into the binding site of tubulin (PDB: 1TUB) with 0.6014 Å of root mean-square deviation between docked and crystallized conformation and a total docking core of 6.3796. (B) **19** docked on to tubulin with high binding affinity, as indicated by a total docking score of 8.6059 compared to taxol (C) **10** docked on tubulin with high binding affinity, as indicated by a total docking score of 6.4471. (D) Podophyllotoxin docked onto tubulin with a low binding affinity, as indicated by a total docking score of 2.9284. In A, B and C structures, tubulin adopts the same active conformation and the inhibitors bind in a similar manner, with enlarged hydrophobic pocket that is characteristic of the active conformation.

affinity,[35] but does not perturb the morphology of microtubules as visible under microscope.[36] In ADME analysis both **10** and **19** exhibited good pharmacokinetics profile for better bioavailability.

Tubulin polymerization kinetics clearly show that compound **10** stabilizes polymerization reaction (Fig. 4). However, at morphological evaluation under confocal microscopy, microtubule condensation in compound **10** treated cells was not as evident as in positive control groups. Hence, we assume that our compound **10** may also act in a different way leading to tubulin stabilization without any morphological changes in microtubule architecture promoting cancer cell death. Nonetheless, presence of dense tubulin in compound **10** treated cells may denote stabilization of microtubule network.

Compound **10** caused significant arrest at G2/M phase arrest, S-phase decrease and increase in sub-diploid population depending on concentration in MCF-7 cells. However, in case of MDA-MB-231 cells, it did not affect any phase of cell cycle in MDA-MB-231, but only increased sub-diploid population. We assume that microtubule stabilization action of the lead compound is responsible for subsequent cell cycle arrest and apoptosis in breast cancer cells. Compound **10** is found to be relatively cytotoxic in vitro against normal cells. However, it is well recognized that toxicity in vivo reflects better profile of a compound than in vitro model. Therefore, we carried out in vivo acute oral toxicity studies. In in vivo acute oral toxicity compound **10** was well tolerated up to 300 mg/kg and can be declared as non-toxic up to this level.

Table 2
Comparison of binding affinities of standard drug (control) and most active compounds **10** and **19** with tubulin

Compound name	Total score	Amino acid involved in active pocket in 4 Å	Involved group of amino acids	Length of H-bond Å	No. of hydrogen bonds
Taxol (standard stabilizer, control positive)	6.3796	GLU-22, VAL-23, ILE-24, SER-25, ASP-26, LEU-42, PHE-83, LEU-217, LEU-219, ASP-226, HIS-229, LEU-230, SER-232, ALA-233, SER-236, PHE-272, PRO-274, LEU-275, THR-276, SER-277, ARG-278, GLN-281, ARG-320, PRO-360, ARG-369, GLY-370, LEU-371	ASP-226 THR-276 ARG-278 ARG-369	1.9 1.8 1.9 1.8	4
19	8.6059	VAL-23, CYS-213, LEU-217, LYS-218, LEU-219, ASP-226, HIS-229, LEU-230, SER-232, ALA-233, SER-236, PHE-272, PRO-274, LEU-275, THR-276, SER-277, ARG-278, GLN-281, ARG-320, PRO-360, LEU-371	THR-276 HIS-229	2.0 1.8	2
10	6.4471	LYS-19, GLU-22, VAL-23, ASP-26, LEU-42, PHE-83, HIS-229, SER-232, ALA-233, SER-236, PHE-272, ARG-320, PRO-360, ARG-369, LEU-371,	GLU-22 LYS-19	1.9 1.8	2
Podophyllotoxin (standard inhibitor, control negative)	2.9284	VAL-23, ASP-26, HIS-229, LEU-230, SER-232, ALA-233, SER-236, PHE-272, ARG-320, PRO-360, ARG-369, GLY-370, LEU-371	—	—	—

Table 3
Various physicochemical (ADME) parameters calculated for **10** and **19**

Compound name	Log*S* for aqueous solubility	Log*P*	Log Khsa for serum protein binding	Log BB for brain/blood	No. of metabolic reactions	Predicted CNS Activity	HERG for K^+ ($Log IC_{50}$) channel blockage	Apparent Caco-2 permeability (nm/s)	Apparent MDCK permeability (nm/s)	Log Kp for skin permeability	% Human oral absorption in GI (±20%)	Qual. model for human oral absorption
19	−5.656	4.186	0.535	−2.007	1	−2	−6.278	240.84	106.187	−2.83	85.866	High
10	−5.449	3.855	0.509	−2.211	2	−2	−6.302	154.116	65.541	−3.133	50.034	High
PDT	−5.358	3.317	0.111	0.21	6	1	−3.403	7274.037	4225.005	−1.026	84.428	High
Taxol	−10.117	5.052	0.585	−1.984	8	−2	−6.752	224.696	98.515	−1.889	44.589	Medium
Stand. range*	(−6.5/0.5)	(−2.0/6.5)	(−1.5/1.5)	(−3.0/1.2)	(1.0/8.0)	(−2 Inactive, +2 active)	(Concern below −5)	(<25 Poor, >500 great)	(<25 Poor, >500 great)	(−8.0 to −1.0, Kp in cm/h)	(<25% is poor)	(>80% is high)

* Note: for 95% of known drugs based on Schrödinger, USA—Qikprop v3.3 (2010) software results.

Table 4
Effect of **10** as a single acute oral dose at 5, 50, 300 and 1000 mg/kg body weight on body weight, haemogram and serum biochemical parameters in Swiss albino mice

Parameters	Dose of **10** at mg/kg body weight as a single oral dose				
	Control	5 mg/kg	50 mg/kg	300 mg/kg	1000 mg/kg
Body weight (g)	24.5 ± 0.76	24.33 ± 1.26	24.83 ± 0.65	25.0 ± 1.06	26.50 ± 0.89
Haemoglobin (g/dL)	11.39 ± 0.84	12.35 ± 0.73	10.67 ± 0.54	10.83 ± 1.03	13.32 ± 0.42
RBC (million/mm^3)	5.43 ± 0.27	5.99 ± 0.25	5.58 ± 0.49	5.11 ± 0.37	5.15 ± 0.64
WBC (1000*/mm^3)	14.2 ± 7.49	14.6 ± 6.99	14.7 ± 1.43	13.76 ± 1.83	15.94 ± 1.52
ALKP (U/L)	218.67 ± 12.43	227.47 ± 25.91	234.14 ± 12.24	217.42 ± 15.97	143.89 ± 9.18
SGOT (U/L)	25.27 ± 1.32	27.67 ± 1.26	34.34 ± 4.20	37.95 ± 3.42	53.29 ± 3.67*
SGPT (U/L)	12.28 ± 3.73	13.15 ± 5.22	12.52 ± 1.69	15.56 ± 5.20	21.95 ± 3.98
Albumin (g/dL)	3.35 ± 0.51	3.17 ± 0.12	2.76 ± 0.10	2.59 ± 0.25	3.86 ± 0.78
Creatinine (mg/dL)	0.63 ± 0.18	1.05 ± 0.24	1.45 ± 0.49	0.79 ± 0.14	1.87 ± 0.45
Triglycerides (mg/dL)	121.45 ± 24.02	106.46 ± 13.63	147.23 ± 18.15	134.38 ± 17.99	156.83 ± 29.30
Serum protein (mg/ml)	1.11 ± 0.14	0.88 ± 0.03	0.94 ± 0.10	0.92 ± 0.06	1.02 ± 0.06
Cholesterol (mg/dL)	81.64 ± 4.26	75.94 ± 4.08	58.62 ± 1.96	77.65 ± 6.12	60.30 ± 4.50

Mean ± SE; $n = 6$.
* $P < 0.05$ compared to control, 5, 50, 300, 1000 mg/kg.

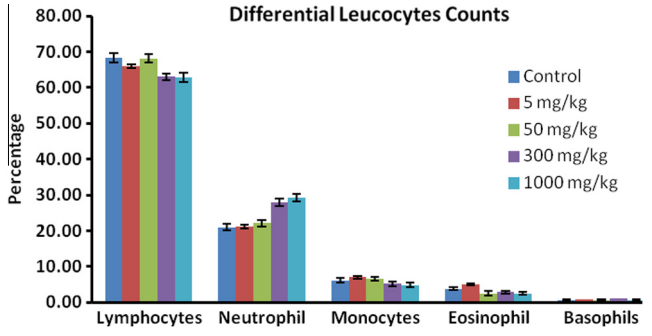


Figure 7. Effect of **10** as a single acute oral dose at 5, 50, 300 and 1000 mg/kg body weight on differential leucocytes counts in Swiss albino mice ($n = 6$, non significant changes were found compared to control).

4. Conclusion

The lignans and neolignans have exhibited significant anti-breast cancer activity. In cell cycle analysis, we observed that compound **10** caused significant enhancement of sub-diploid population that is indicative of increased apoptosis, along with or without arrest of cell cycle phases depending on the concentrations. As suggested by in silico studies and biochemical tubulin polymerization assay, the probable mechanism of action of the lead compound **10** and **19** is through stabilization of microtubule assembly. Compound **10** was found to be non-toxic up to 300 mg/kg dose in Swiss albino mice. These neolignan lead molecules may further be optimized to get better anticancer agent.

5. Experimental section

5.1. General methods

Reagents and biological standards, MTT, podophyllotoxin, taxol, etc. were procured from Sigma–Aldrich, USA and used as such without purification. Melting points were determined on E-Z melt automated melting point apparatus Stanford Research System, USA in open capillaries. For thin layer chromatography, Merck silica gel (TLC, UV_{254nm}) aluminium sheets were used to monitor the reactions and visualisation was done by spraying 2% ceric sulfate–10% aqueous sulfuric acid subsequent charring at 80–100 °C. Dry solvents were prepared as per standard methods. Purifications of compounds were done through column chromatography over silica gel (60–120 and 100–200 mesh, Thomas Baker), evaporating the solvents under reduced pressure. NMR experiments were carried out on Bruker Avance DRX-300 MHz instrument using tetramethylsilane (TMS) as internal standard. Chemical shifts are given in δ ppm. NMR abbreviations for signal patterns are as; s, singlet; d, doublet; t, triplet, m, multiplet and br s, broad singlet. All ^1H and few ^{13}C spectral data are reported. Electrospray ionization mass spectra (ESI-MS) were recorded on APC3000 (Applied biosystem) LC–MS–MS after dissolving compounds in methanol. FT-IR spectra were recorded on Perkin–Elmer SpectrumBX after making KBr pellets. Tubulin polymerisation assay kit (BK006P) was procured from Cytoskeleton, USA. The nomenclatures of lignans and neolignans have been given as per IUPAC recommendations.[37]

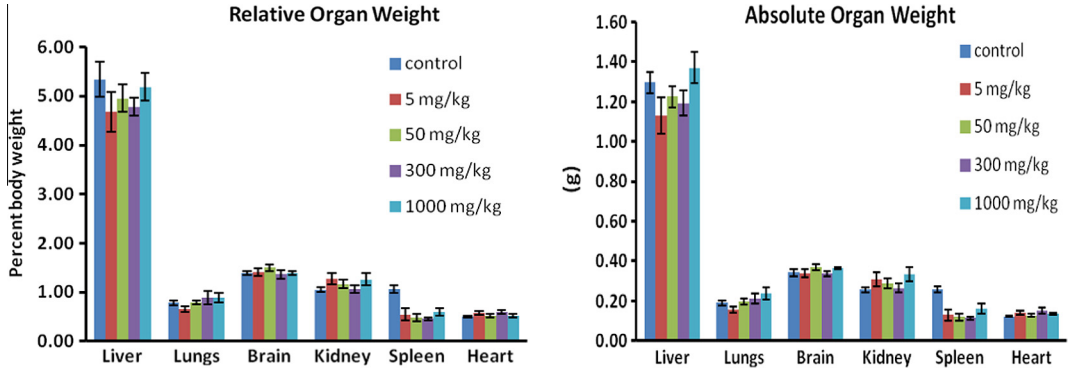


Figure 8. Effect of **10** as a single acute oral dose at 5, 50, 300 and 1000 mg/kg on absolute and relative organ weight in Swiss albino mice (n = 6, non significant changes were found compared to control).

5.2. Chemical synthesis

5.2.1. General procedure for the synthesis of esters 2, 8 and 9

5.2.1.1. Synthesis of 3,4,5-trimethoxycinnamic acid ethyl ester (2). Compound **1** (1 g, 3.94 mmol) was taken in 2 mL of thionyl chloride. To this stirred solution absolute ethanol (2 mL) was added and further stirred for 2 h at room temperature. Excess of thionyl chloride and ethanol were removed under reduced pressure and the reaction mixture was quenched with water. The product was extracted with ethyl acetate, washed with water and dried over anhydrous sodium sulfate. The organic layer was evaporated to dryness to get a crude residue. It was purified through column chromatography over silica gel (60–120 mesh) to get the desired ester as white crystalline solid.

Alternatively, these products can also be obtained using another protocol. Same quantity of acid (1 g) was taken in 15 mL ethanol. To this 0.5 mL concentrated sulfuric acid was added and the reaction mixture was refluxed for 4 h. The solvent was evaporated and 10 mL water was added to it. The reaction mixture was extracted with ethyl acetate, washed with water and dried over anhydrous sodium sulfate. The solvent was evaporated and the crude product was purified through column chromatography over silica gel to get the desired ester **2**.

Yield 91%; mp = 68–70 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.32 (t, 3H, CH_3), 3.87 (s, 9H, 3× OCH_3), 4.24 (q, 2H, OCH_2), 6.31–6.36 (d, 1H, =CH, J = 15.9 Hz), 6.74 (s, 2H, CH, aromatic), 7.56–7.61 (d, 1H, CH=, J = 15.9 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.72, 56.52, 56.52, 60.88, 61.34, 105.56, 105.56, 117.90, 130.35, 140.42, 144.95, 153.81, 153.81, 167.33; Electrospray mass: 289.2 $[M+Na]^+$, 305.2 $[M+K]^+$, 555.3 $[2M+Na]^+$. IR (cm^{-1}): 2943, 1702, 1633, 1583, 1506, 1279, 1122.

5.2.1.2. 4-Hydroxy cinnamic acid ethyl ester (8). Yield 94%; mp = 69–71 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.33 (t, 3H, CH_3), 4.28 (m, 2H, OCH_2), 6.26–6.31 (d, 1H, =CH, olefinic, J = 15.9 Hz), 6.86–6.89 (d, 1H, CH, aromatic, J = 8.4 Hz), 7.38–7.41 (d, 1H, CH, aromatic, J = 8.4 Hz), 7.61–7.66 (d, 1H, CH=, olefinic, J = 15.9 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.69, 61.26, 115.32, 116.41, 116.41, 127.04, 130.50, 130.50, 145.61, 158.91, 168.86. Electrospray mass (MeOH): 193 $[M+H]^+$, negative mode: 191 $[M-H]^-$; IR (cm^{-1}): 3289, 1717, 1683, 1604, 1516, 1280.

5.2.1.3. 4-Hydroxy-3-methoxycinnamic acid ethyl ester (9). Yield 89%; mp = 61–63 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.22 (t, 3H, CH_3), 3.88 (s, 3H, OCH_3), 4.25 (q, 2H, OCH_2), 6.22 (d, 1H, OH, phenolic), 6.24–6.29 (d, 1H, =CH—CO—, J = 15.9 Hz), 6.89 (d, 1H, CH, aromatic), 7.05 (d, 2H, CH, aromatic), 7.57–7.62 (d, 1H, CH=, J = 15.9 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.72, 56.30, 60.80, 109.85, 115.23, 115.90, 123.39, 127.35, 145.18, 147.28, 148.43, 167.81; Electrospray mass (MeOH): 245 $[M+Na]^+$, Negative mode: 221 $[M-H]^-$; IR (cm^{-1}): 3399, 2979, 1707, 1688, 1602, 1516, 1270, 1176.

5.2.2. Synthesis of 4-hydroxy-3,5-dimethoxycinnamic acid ethyl ester (3)

The demethylation of **2** was done as per previously reported method.[29]

5.2.2.1. 4-Hydroxy-3,5-dimethoxycinnamic acid ethyl ester (3). Yield 84%; mp = 74–77 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.33 (t, 3H, CH_3), 3.90 (s, 6H, 2× OCH_3), 4.24 (q, 2H, OCH_2), 5.81 (s, 1H, exchangeable, phenolic OH), 6.27–6.32 (d, 1H, =CH, J = 15.9 Hz), 6.76 (s, 2H, CH, aromatic), 7.56–7.61 (d, 1H, CH=, J = 15.6 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.74, 56.71, 56.71, 60.80, 105.40, 105.40, 116.41, 126.34, 137.47, 145.27, 147.60, 147.60, 167.57. Electrospray mass: 253.3 $[M+H]^+$, 275.1 $[M+Na]^+$, 291.1 $[M+K]^+$, 527.2 $[2M+Na]^+$; IR (cm^{-1}): 3371, 2940, 1693, 1601, 1516, 1190, 1110.

5.2.3. General procedure for the synthesis of lignan 5a and neolignans 4, 10 and 11

5.2.3.1. Synthesis of neolignan 4 and lignan 5a. To a stirred cold solution of aqueous KOH (6%, 20 mL) a solution of ester **3** (504 mg, 2.0 mmol) in benzene (15 mL) was added. After 10 min potassium ferricyanide (329 mg, 0.1 mmol) was added to this reaction mixture to give a light brown colour. After 30 min when the reaction mixture turns to light green, it is quenched with dil HCl (5%, 5 mL). It was extracted with ethyl acetate (3 × 20 mL), washed with water and dried over anhydrous sodium sulfate. The organic layer was dried in vacuo. The residue thus obtained was purified through column chromatography over silica gel to get neolignan (**4**) at 6% ethylacetate–hexane and lignan (**5a**) at 9% ethyl acetate–hexane both as dense liquids.

5.2.3.2. Diethyl-4,4′-dihydroxy-3,3′,5,5′-tetramethoxy-8,2′-neolign-7,7′-dien-9,9′-dionate (4). Yield 21%; oil, ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.18 (bs, 3H, CH_3), 1.34 (t, 3H, CH_3), 3.85 (s, 6H, OCH_3), 4.06 (s, 6H, 2× OCH_3), 4.38 (m, 4H, 2× OCH_2), 5.8 (bs, 1H, exchangeable, phenolic OH), 6.1 (bs, 1H, exchangeable, phenolic OH), 6.28–6.33 (d, 1H, =CH, J = 15.9 Hz), 6.78 (s, 1H, CH, aromatic), 7.01 (s, 1H, CH, aromatic), 7.57–7.62 (d, 1H, CH=, J = 15.9 Hz), 8.09 (s, 1H, CH, aromatic), 8.37 (s, 1H, CH, aromatic). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.58, 14.74, 56.71, 56.71, 60.79, 61.67, 61.87, 61.87, 102.67, 102.67, 105.46, 105.46, 116.46, 123.56, 125.18, 127.74, 127.74, 128.75, 129.19, 139.46, 139.46, 145.25, 147.63, 147.63, 168.33, 168.67. Electrospray mass: 503.4

$[M+H]^+$, 525.3 $[M+Na]^+$, 541.4 $[M+K]^+$. IR (cm^{-1}): 3401, 2933, 1718, 1628, 1477, 1272, 1107.

5.2.3.3. Diethyl-4,4′-dihydroxy-3,3′,5,5′-tetramethoxy-lign-7,7′-dien-9,9′-dionate (5a). Yield 29%; gummy, ^1H NMR ($CDCl_3$, 300 MHz): δ 1.20 (t, 6H, 2× CH_3), 3.63 (s, 3H, OCH_3), 3.72 (s, 6H, 2× OCH_3), 3.88 (s, 3H, OCH_3), 4.02 (m, 4H, 2× OCH_2), 4.96 (s, 2H, CH=, olefinic), 5.36 (bs, 1H, exchangeable, OH), 5.81 (bs, 1H, exchangeable, OH), 6.27 (s, 2H, CH, aromatic), 6.68 (s, 1H, CH, aromatic), 7.60 (s, 1H, CH, aromatic). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.44, 14.61, 56.61, 56.61, 56.61, 60.95, 61.09, 61.57, 104.81, 104.81, 107.81, 107.81, 123.66, 123.78, 124.21, 133.96, 134.11, 137.67, 137.67, 141.37, 145.39, 147.21, 147.21, 167.13, 172.40. Electrospray mass: 525.3 $[M+Na]^+$, 541.3 $[M+K]^+$. IR (cm^{-1}): 3401, 2929, 1720, 1703, 1687, 1596, 1496, 1258.

5.2.3.4. Diethyl-4,4′-dihydroxy-8,3′-neolign-7,7′-dien-9,9′-dionate (10). Yield 24%, oil, ^1H NMR ($CDCl_3$, 300 MHz): δ 1.30 (t, 6H, 2× CH_3), 4.28 (q, 4H, 2× OCH_2), 6.07 (d, 1H, CH, aromatic, J = 7.5 Hz), 6.27–6.32 (d, 1H, =CH, olefinic, J = 16.2 Hz), 6.82 (m, 3H, 3× CH, aromatic), 7.23 (d, 2H, 2× CH, aromatic), 7.44 (d, 1H, CH, aromatic, J = 9.6 Hz), 7.57 (s, 1H, CH, olefinic), 7.59–7.64 (d, 1H, CH=, olefinic, J = 16.2 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.67, 14.73, 61.00, 62.37, 110.71, 115.89, 116.11, 116.11, 125.31, 125.67, 127.92, 127.92, 128.20, 130.26, 131.14, 132.27, 145.05, 156.75, 156.75, 161.56, 168.13, 170.89; Electrospray mass (MeOH): 421 $[M+K]^+$, Negative mode: 381 $[M-H]^-$; ESI-HRMS: 383.1489 (calcd for $C_{22}H_{23}O_6$, 383.1495), 405.1307 (calcd for $C_{22}H_{22}O_6Na$, 405.1314), 421.1052 (calcd for $C_{22}H_{22}O_6K$, 421.1054); IR: 3393, 2980, 2928, 1733, 1706, 1602, 1511, 1245.

5.2.3.5. Diethyl-4,4′-dihydroxy-5,5′-dimethoxy-8,3′-neolign-7,7′-dien-9,9′-dionate (11). Yield 26%, mp = 127–29 °C; ^1H NMR ($CDCl_3$, 300 MHz): δ 1.34 (t, 6H, 2× CH_3), 3.87 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 4.29 (q, 4H, 2× OCH_2), 5.27 (s, 2H, 2× OH, both phenolic OH), 6.10 (d, 1H, CH aromatic, J = 6.9 Hz), 6.27–6.33 (d,1H, CH=, olefinic, J = 15.9 Hz), 6.90 (s, 3H, 3× CH, aromatic), 7.02 (s, 1H, CH, aromatic), 7.18 (s, 1H, CH=, olefinic), 7.61–7.66 (d, 1H, olefinic, J = 15.9 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.69, 14.75, 55.97, 56.42, 60.84, 62.30, 87.91, 109.16, 112.29, 114.92, 116.34, 118.30, 119.90, 126.26, 129.03, 131.87, 144.97, 145.12, 146.45, 146.45, 147.12, 150.32, 167.67, 170.66. Electrospray mass (MeOH): 465 $[M+Na]^+$; Negative mode: 441 $[M-H]^-$; IR (cm^{-1}): 3422, 2934, 1737, 1718, 1606, 1520, 1270, 1172.

5.2.4. General procedure for the synthesis of lignans 17 and 18 through Stobbe's condensation

5.2.4.1. Diethyl-3,3′,4,4′,5,5′-hexamethoxy-lign-7,7′-dien-9,9′-dionate (18). Pre-washed sodium hydride (138 mg, 5.75 mmol) was stirred in dry DMF (5 mL) at room temperature under inert atmosphere (N_2). To this diethyl succinate (200 mg, 1.15 mmol) and 3,4,5-trimethoxybenzaldehyde (271 mg, 1.38 mmol) were added as solution in DMF (5 mL) through syringe. The reaction mixture was refluxed at 100 °C for 6 h. On completion, the reaction mixture was cooled and quenched with dil HCl (5%, 10 mL). It was extracted with ethyl acetate (3 × 25 mL), washed with water and dried over anhydrous sodium sulfate and evaporated to dryness. The residue thus obtained was a complex mixture and was difficult to purify. So, the crude mass thus obtained was as such processed for esterification with diethyl sulfate in potassium carbonate-DMSO/acetone (1:3) system. After routine work-up, it was purified through column chromatography over silica gel (100–200 mesh) to get **18** as viscous oil.

Compound **18**: Yield 49%; oil, ^1H NMR ($CDCl_3$, 300 MHz): δ 1.25 (t, 6H, 2× CH_3), 3.81 (s, 12H, 4× OCH_3), 3.92 (s, 6H, 2× OCH_3), 4.13–4.20 (m, 4H, OCH_2), 6.77 (s, 4H, CH, aromatic), 7.82 (s, 2H, olefinic, CH). Electrospray mass: 531.2 $[M+H]^+$, 553.3 $[M+Na]^+$, 569.3 $[M+K]^+$; IR (cm^{-1}): 2925, 1772, 1764, 1601, 1458, 1256, 1187, 1129.

5.2.4.2. Diethyl-3,3′,4,4′-tetramethoxy-lign-7,7′-dien-9,9′-dionate (17). Yield 43%, oil, ^1H NMR ($CDCl_3$, 300 MHz): δ 1.24 (t, 6H, 2× CH_3), 3.70 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 4.24 (m, 4H, 2× OCH_2), 6.77–6.88 (m, 6H, aromatic), 7.56 (s,1H, olefinic, CH=), 7.82 (s, 1H, olefinic, CH=). Electrospray mass: 493.3 $[M+Na]^+$, 509.2 $[M+K]^+$; IR (cm^{-1}): 2933, 1702, 1596, 1514, 1257.

5.2.5. Synthesis of 5b, 19, 20, 28 were carried out as per reported method[30]

5.2.5.1. Diethyl-4,4′-diacetoxy-3,3′,5,5′-tetramethoxy-lign-7,7′-dien-9,9′-dionate (5b). Yield 92%, oil, ^1H NMR ($CDCl_3$, 300 MHz): δ 1.27 (bs, 6H, 2× CH_3), 2.27 (s, 3H, OAc), 2.29 (s, 3H, OAc), 3.64 (s, 3H, OCH_3), 3.68 (s, 6H, 2× OCH_3), 3.88 (s, 3H, OCH_3), 4.16 (bm, 4H, 2× OCH_2), 6.30 (s, 2H, 2× CH, aromatic), 6.77 (s, 1H, =CH), 7.28 (bs, 1H, CH=), 7.61 (s, 2H, 2× CH, aromatic). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.44, 14.63, 20.86, 23.09, 56.45 (×3), 56.60, 61.38, 61.76, 104.52 (×4), 108.55, 114.47, 123.13, 126.63, 127.80, 130.28, 136.58, 136.58, 140.90, 151.34, 152.27, 152.27, 166.69, 168.48, 169.12, 171.93. Electrospray mass: 609.2 $[M+Na]^+$, 625.3 $[M+K]^+$. IR (cm^{-1}): 2926, 1766, 1720, 1705, 1601, 1188.

5.2.5.2. Diethyl-4-acetoxy-4′-hydroxy-8,3′-neolign-7,7′-dien-9,9′-dionate (19). Yield 89%, mp = 65–68 °C; ^1H NMR ($CDCl_3$, 300 MHz): δ 1.31 (t, 6H, 2× CH_3), 2.24 (s, 3H, OAc) 4.24 (m, 4H, 2× OCH_2), 6.12 (d, 1H, CH, aromatic, J = 7.5 Hz), 6.23–6.28 (d, 1H, =CH, J = 16.2 Hz), 6.86 (d, 1H, CH, aromatic, J = 8.4 Hz), 7.05 (d, 2H, CH, aromatic, J = 8.4 Hz), 7.44 (m, 3H, 3× CH, aromatic), 7.52 (s, 1H, CH, olefinic), 7.55–7.61 (d, 1H, CH=, J = 16 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.70, 14.76, 21.50, 60.80, 62.40, 110.72, 116.22, 122.43, 122.43, 126.79, 126.79, 127.34, 127.34, 128.48, 130.20, 131.07, 132.03, 138.16, 144.66, 151.11, 161.41, 167.69, 169.80, 170.57. Electrospray mass: 463 $[M+K]^+$; ESI-HRMS: 425.1591 (calcd for $C_{24}H_{25}O_7$, 425.1600), 447.1410 (calcd for $C_{24}H_{24}O_7Na$, 447.1420), 463.1156 (calcd for $C_{24}H_{24}O_7K$, 463.1159), calcd: 491.24099; IR (cm^{-1}): 3449, 2928, 1737, 1719, 1702, 1654, 1603, 1233.

5.2.5.3. Diethyl-4-acetoxy-4′-hydroxy-5,5′-dimethoxy-8,3′-neolign-7,7′-dien-9,9′-dionate (20). Yield 92%, mp = 106–108 °C; ^1H NMR ($CDCl_3$, 300 MHz): δ 1.31 (t, 6H, 2× CH_3), 2.26 (s, 3H, O–CO–CH_3), 3.80 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 4.26 (q, 4H, 2× OCH_2), 6.16 (d, 1H, CH, aromatic, J = 7.8 Hz), 6.25–6.31 (d, 1H, CH=, olefinic, J = 15.9 Hz), 6.88–7.01 (m, 4H, aromatic proton), 7.31 (s, 1H, CH=, olefinic), 7.59–7.64 (d, 1H, =CH, olefinic, J = 15.9 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.69, 14.69, 21.03, 56.39, 56.58, 60.82, 62.39, 110.54, 110.54, 112.34, 116.53, 118.32, 118.66, 123.48, 126.03, 129.27, 139.03, 140.20, 144.86, 145.15, 147.20, 148.10, 151.73, 168, 169, 170.51; Electrospray mass (MeOH): 507 $[M+Na]^+$. IR (cm^{-1}): 3449, 2983, 2930, 1768, 1734, 1702, 1609, 1275.

5.2.5.4. Diethyl-4,4′,5,5′-tetramethoxy-8,3′-neolign-7,7′-dien-9,9′-diacetate (24). Yield 91%, oil, ^1H NMR ($CDCl_3$, 300 MHz): δ 2.00, 2.02 (s, 6H, 2× CH_3), 3.51 (s, 3H, OCH_3), 3.66 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3), 3.78 (s, 3H, OCH_3), 4.61–4.64 (m, 2H, CH_2), 4.80 (s, 2H CH_2), 6.51–6.74 (m, 8H, aromatic, and olefinic). ^{13}C NMR ($CDCl_3$, 75 MHz) 21.36, 21.36, 55.52, 56.04, 56.23, 61.08, 64.16, 65.47, 108.61, 111.38, 112.76, 119.01, 120.19, 121.42, 122.78, 132.37, 132.42, 133.15, 134.55, 135.52, 147.09, 147.72, 148.93, 154.01, 170.08, 171.44. Electrospray mass (MeOH):

493 $[M+Na]^+$, 495.5 $[M+Na+2]^+$, 497 $[M+Na+4]^+$. IR (cm^{-1}): 2931, 2839, 1735, 1720, 1704, 1687, 1595, 1513, 1461, 1252, 1159, 1024.

5.2.6. General procedure of synthesis of 21 and 22

The procedure followed is same as described at Section 5.2.1.

5.2.6.1. Diethyl-4,4′-dimethoxy-8,3′-neolign-7,7′-dien-9,9′-dionate (21). Yield 91%, gummy, ^1H NMR ($CDCl_3$, 300 MHz): δ 1.26 (t, 6H, 2× CH_3), 3.78 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 4.22 (m, 4H, 2× OCH_2), 6.21–6.26 (d, 1H, =CH, J = 15.9 Hz), 6.69 (m, 2H, CH, aromatic, J = 8.7 Hz), 7.02 (m, 4H, 4× CH, aromatic), 7.52 (s, 1H, CH, aromatic), 7.56–7.61 (m, 1H, CH=, J = 16.5 Hz), 7.81 (s, 1H, CH=). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.71, 14.71, 55.61, 56.18, 60.71, 61.27, 111.70, 114.20, 114.20, 116.39, 126.74, 127.64, 127.87, 130.13, 131.29, 132.34, 132.34, 140.84, 144.49, 159.79, 160.71, 160.71, 167.68, 168.14. Electrospray mass: 411.3 $[M+H]^+$, 433.2 $[M+Na]^+$, 449.2 $[M+K]^+$, 843.5 $[2M+Na]^+$, 1253.6 $[3M+Na]^+$; IR (cm^{-1}): 2978, 1706, 1602, 1510, 1254, 1174.

5.2.6.2. Diethyl-4,4′,5,5′-tetramethoxy-8,3′-neolign-7,7′-dien-9,9′-dionate (22). Yield 87%, gummy, ^1H NMR ($CDCl_3$, 300 MHz): δ 1.28 (t, 6H, 2× CH_3), 3.45 (s, 3H, OCH_3), 3.74 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 4.26 (q, 4H, 2× OCH_2), 6.26–6.32 (d, 1H, =CH, olefinic, J = 15.9 Hz), 6.53 (s, 1H, CH, aromatic), 6.72 (d, 1H, CH, aromatic, J = 8.1 Hz), 6.84 (d, 1H, CH, aromatic, J = 8.4 Hz), 6.94 (s, 1H, CH, aromatic), 7.09 (s, 1H, aromatic), 7.54–7.59 (d, 1H, olefinic, CH=, J = 15.9 Hz), 7.80 (s, 1H, CH, olefinic); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.73, 14.73, 55.51, 56.18, 56.30, 60.89, 61.01, 61.50, 111.05, 111.31, 112.47, 117.92, 124.08, 125.64, 126.65, 127.63, 131.09, 131.86, 141.06, 144.30, 148.71, 149.59, 150.52, 153.62, 167.31, 168. Electrospray mass (MeOH): 493.2 $[M+Na]^+$, 509.2 $[M+K]^+$; IR (cm^{-1}): 2937, 1706, 1635, 1599, 1513.

5.2.7. General procedure of synthesis of amides 23 and 26

5.2.7.1. 4,4′-Dihydroxy-5,5′-dimethoxy-8,3′-neolign-7,7′-dien-9,9′-diamide (23). Neolignan **11** (110 mg, 0.25 mmol) was stirred in 30% aqueous ammonia (2 mL) with cooling at 0–10 °C for an hour. The reaction mixture was stirred overnight (16–18 h) at room temperature. The reaction mixture was quenched with dil HCl (5%, 10 mL) and extracted with ethyl acetate, washed with water and dried over anhydrous sodium sulfate. The organic layer was dried in vacuo to get a residue. The residue was purified through column chromatography over silica gel (60–120 mesh). The desired amide **23** was obtained as viscous liquid.

Compound **23**: Yield 58%, oil, ^1H NMR ($CDCl_3$, 300 MHz): δ 3.47 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 5.80 (bs, NH), 6.02 (bs, NH), 6.23–6.28 (d, 1H, =CH, olefinic, J = 15.9 Hz), 6.56 (s, 1H, CH, aromatic), 6.74–6.76 (m, 2H, CH, aromatic), 6.96 (s, 1H, CH, aromatic), 7.04 (s, 1H, CH, aromatic), 7.54–7.59 (d, 1H, CH=, olefinic, J = 15.9 Hz), 7.83 (s, 1H, olefinic, =CH). Electrospray mass (MeOH): 385 $[M+H]^+$, 397 $[M+Na]^+$, Negative mode: 383 $[M-H]^-$; IR: 3369, 2924, 1702, 1655, 1595, 1494, 1271.

5.2.7.2. 4,4′,5,5′-Tetramethoxy-8,3′-neolign-7,7′-dien-9,9′-diamide (26). Yield 62%, oil, ^1H NMR ($CDCl_3$, 300 MHz): δ 3.38 (s, 3H, OCH_3), 3.75 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 6.19–6.33 (bp, 4H, 2× NH_2), 6.46 (s, 1H), 6.63–6.65 (d, 1H), 6.74–6.77 (d, 1H), 6.87–6.89 (s, 2H), 7.12–7.18 (d, 1H), 7.58–7.60 (d, 1H), 7.73 (s, 1H, aromatic). Electrospray mass (MeOH): 413 $[M+H]^+$, 451 $[M+K]^+$; IR (cm^{-1}): 3425, 3400, 2927, 1703, 1686, 1595, 1582, 1512, 1253, 1147.

5.2.8. 4,4′,5,5′-Tetramethoxy-8,3′-neolign-7,7′-dien-9,9′-diol (27)

Diester **22** (100 mg, 0.21 mmol) was stirred in 10 mL THF at 0 °C. To this, lithium borohydride (120 mg, 5.71 mmol) was added and stirred for an hour. After this it was further stirred at room temperature for 3 h. On completion, it was quenched by adding dil HCl (5%, 10 mL), extracted with ethyl acetate, washed with water and dried over anhydrous sodium sulfate. The organic layer was dried in vacuo to get a residue. It was recrystallised with $CHCl_3$–Pet ether (1:4) to get a gummy solid.

Compound **27**: Yield 87%, gummy, ^1H NMR ($CDCl_3$, 300 MHz): δ 3.54 (s, 3H, OCH_3), 3.61 (s, 3H, OCH_3), 3.80 (s, 6H, 2× OCH_3), 4.17–4.23 (m, 2H, OCH_2), 4.32 (s, 2H, OCH_2), 6.17–6.87 (m, 8H, aromatic and olefinic). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 55.49, 56.00, 56.08, 56.23, 62.19, 62.34, 108.4, 111.08, 111.42, 111.75, 112.86, 118.74, 119.82, 122.52, 127.77, 130.44, 135.63, 147.55, 148.42, 148.88, 152.94, 153.19. Electrospray mass (MeOH): 409 $[M+Na]^+$, 425 $[M+K]^+$. IR (cm^{-1}): 3426, 2926, 1654, 1636, 1628, 1460, 1438, 1144, 1024.

5.2.9. 4,4′,5,5′-Tetramethoxy-8,3′-neolign-7,7′-dien-9,9′-dioic acid (25)

Diester **22** (42 mg, 0.09 mmol) was stirred in 5% aqueous methanolic (1:4) KOH (10 mL). The reaction mixture was heated to 50 °C for 2 h. Reaction was neutralized with dil HCl (5%, 10 mL), extracted with ethyl acetate, washed with water and dried over anhydrous sodium sulfate. Solvent was evaporated to dryness and the residue thus obtained was crystallized from methanol to get **25**.

Yield 86%, oil, ^1H NMR ($CDCl_3$, 300 MHz): δ 3.47 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 6.28–6.33 (d, 1H, =CH, olefinic, J = 15.9 Hz), 6.56 (s, 1H, CH, aromatic), 6.74 (d, 1H, CH, aromatic, J = 7.4 Hz), 6.86 (d, 1H, CH, aromatic, J = 8.1 Hz), 6.99 (s, 1H, CH, aromatic), 7.13 (s, 1H, CH, aromatic), 7.64–7.69 (d, 1H, CH=, J = 15.9 Hz), 7.92 (s, 1H, CH, olefinic). ^{13}C NMR ($CDCl_3$, 75 MHz): 55.56, 56.23, 56.33, 61.14, 62.00, 111.09, 111.20, 111.69, 117.05, 119.01, 125.20, 127.24, 130.78, 148.80, 150.03, 172.02, 173.01. Electrospray mass (MeOH): 437.2 $[M+Na]^+$, 453.1 $[M+K]^+$, Negative mode: 413.3 $[M-H]^-$; IR (cm^{-1}): 3448, 2934, 1686, 1514, 1263, 1147.

5.3. Bioevaluation

5.3.1. Cell culture

Human breast cancer cell lines, MCF-7 and MDA-MB-231 were originally obtained from American type of cell culture collection (ATCC), USA and stocks are maintained in laboratory. HEK-293 cells were obtained from institutional cell repository of animal tissue culture facility (CSIR-CDRI). Cells were grown in tissue culture flasks in DMEM (Dulbecco modified Eagle medium, Sigma) supplemented with 10% fetal bovine serum with 1× stabilized antibiotic–antimycotic solution (Sigma) in a CO_2 incubator (Sanyo, Japan) at 37 °C with 5% CO_2 and 90% relative humidity. The cells at subconfluent stage were harvested with 1× porcine pancreatic trypsin (Sigma) and seeded in required density in tissue culture plates for assay.

5.3.2. In vitro cell inhibition assay

The cell inhibiting activity of the compounds of the series were determined using MTT assay as described earlier.[38] In brief, cells were seeded @ 1×10^4 cells/well in each well of 96-well microculture plates in 200 μL DMEM (Sigma), supplemented with 10% FBS and 1× stabilized antibiotic–antimycotic solution (Sigma) and incubated for 24 h at 37 °C in a CO_2 incubator. Compounds, diluted to the desired concentrations in culture medium DMEM without phenol red, supplemented with 0.5% FBS, were added to the wells with respective vehicle control. After 24 h of incubation, 20 μL of 5 mg/mL MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) (Sigma) was added to each well and the plates were further incubated for 3 h. Then the supernatant from each well was

carefully removed without disturbing the formazan crystals. The formazan crystals were dissolved in 200 μL of dimethyl sulfoxide (DMSO) using plate shaker (Biosan) and absorbance at 570 nm wavelength was recorded in a microplate reader (Microquant; BioTek). The cell inhibition of analogues **10** and **19** was also evaluated at 48 and 72 h incubation. Analogues **10** and **19** were also evaluated for toxicity against human embryonic kidney cells, that is, HEK-293. Podophyllotoxin (PDT) and tamoxifen (TAM) were used as positive controls.

5.3.3. Cell cycle analysis

Cell cycle analysis using PI-staining of cells was carried out as per earlier reported method.[39] MCF-7 and MDAMB-231 cells were plated and treated with 10 μM of compound **10** for 24 h, and harvested. For flowcytometry analysis, collected cells were washed with cold PBS, re-suspended at the rate of 2×10^6 cells/ml, fixed in absolute ethanol, treated with RNase A (10 mg/mL), and then stained with propidium iodide (50 μg/mL; Sigma, St. Louis, MO, USA) for 30 min at room temperature. The DNA content of the cells was measured using a FACS Calibur flow cytometer (Becton–Dickinson, San Jose, CA, USA) and CellQuest software.

5.3.4. Tubulin polymerisation assay

Tubulin polymerization experiment was done as per reported method using 'assay kit' from Cytoskeleton, USA.[40] In brief, tubulin protein (3 mg/mL) in tubulin polymerization buffer (80 mM PIPES, pH 6.9, 2 mM $MgCl_2$, 0.5 mM EGTA, 1 mM GTP and 15% glycerol) was placed in pre-warmed 96-well microtiter plates at 37 °C in the presence of test compounds with variable concentrations. All samples were mixed well and polymerization was monitored kinetically at 340 nm every min for 1 h using Spectramax plate reader. Podophyllotoxin (PDT) was used as standard inhibitor of tubulin polymerisation, while paclitaxel (taxol) was used as standard stabilizer of tubulin polymerisation. DMSO was used as negative control.

5.3.5. In silico studies

5.3.5.1. Molecular docking studies. To find the possible bioactive conformations of **10** and **19**, the Sybyl X 1.3 interfaced with Surflex–Dock module was used for molecular docking. Program automatically docks ligand into binding pocket of a target protein by using protomol-based algorithm and empirically produced scoring function. The X-ray crystallographic structures of tubulin complex with ligand (taxol) [PDB: 1TUB][41] was taken from the protein data bank (PDB) and modified for docking calculations. Co-crystallized ligand was removed from the structure, water molecules were removed, H-atoms were added and side chains were fixed during protein preparation. Protein structure minimization was performed by applying Tripos force field and partial atomic charges were calculated by Gasteiger–Huckel method. In reasonable binding pocket, all the compounds were docked into the binding pocket and 20 possible active docking conformations with different scores were obtained for each compound. During the docking process, all of the other parameters were assigned their default values.[31,32]

5.3.5.2. Screening for pharmacokinetic properties. As we know that most of drugs in discovery process fail to cross clinical trials because of poor pharmacokinetic profile (PK). The properties of PK such as absorption, distribution, metabolism, excretion, and toxicity (ADMET) are important descriptors for human therapeutic use of any compound. The compound distribution in human depends on factors, for example, blood–brain barrier (Log BB), permeability (apparent Caco-2 and MDCK permeability, Log Kp for skin permeability), the volume of distribution and plasma protein binding refer by Log Khsa for serum protein binding.[31] These ADMET descriptors were calculated and checked for compliance with their standard ranges. The octanol–water partition coefficient (Log *P*) has been implicated in Log BB penetration and permeability studies. The descriptor values of 90% orally active compounds follows Lipinski's rule. This descriptor has been shown to correlate well with passive molecular transport through membranes. Calculations of other important ADME properties of **10** and **19** were performed through QikProp, version 3.2, Schrödinger, LLC, USA (2010). Compounds **10** and **19** were also screened for Log *S*, Caco-2 and number of primary metabolites (Schrödinger, USA, 2010).

5.3.6. In vivo acute oral toxicity

In view of potent anti-cancer activity of lignan **10** in in vitro model, acute oral toxicity of the same was carried out in Swiss albino mice. Experiment was conducted in accordance with the Organization for Economic Co-operation and Development (OECD) test guideline No. 423 (1987).

For this study 30 mice (15 male and 15 female) were taken and divided into four groups comprising 3 male and 3 female mice in each group weighing between 20 and 25 g. The animals were maintained at 22 ± 5 °C with humidity control and also on an automatic dark and light cycle of 12 h. The animals were fed with the standard mice feed and provided ad libitum drinking water. Mice of group 1 were kept as control and animals of groups 2, 3, 4 and 5 were kept as experimental. The animals were acclimatized for 7 days in the experimental environment prior to the actual experimentation. Neolignan **10** was solubilized in dimethyl sulfoxide and then suspended in caboxymethyl cellulose (0.7%) and was given at 5, 50, 300 and 1000 mg/kg body weight to animals of groups 2, 3, 4 and 5, respectively, once orally. Control animals received only vehicle.

The animals were checked for mortality and any signs of ill health at hourly interval on the day of administration of drug and there after a daily general case side clinical examination was carried out including changes in skin, mucous membrane, eyes, occurrence of secretion and excretion and also responses like lachrymation, pilo-erection, respiratory patterns etc. Also changes in gait, posture and response to handling were also recorded.[42] In addition to observational study, body weights were recorded and blood and serum samples were collected from all the animals on 7th day of the experiment in acute oral toxicity. The samples were analysed for total RBC, WBC, differential leucocytes count, haemoglobin percentage and biochemical parameters like ALKP, SGPT, SGOT, total cholesterol, triglycerides, creatinine, bilirubin, serum protein, tissue protein, malonaldehyde and reduced GSH activity. The animals were then sacrificed and were necropsed for any gross pathological changes. Weights of vital organs like liver, heart, kidney, etc. were recorded.[43]

5.3.7. Statistical analysis

Data were expressed as means. Statistical analysis was done by ANOVA and Newman Keul's test. A value of P values <0.05 were considered a statistically significant difference.

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Supplementary data

Supplementary data (^{1}H NMR, ^{13}C NMR, and Mass of compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.12.067.

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