Synthesis of pharmacologically important naphthoquinones and anticancer activity of 2-benzyllawsone through DNA topoisomerase-II inhibition

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ABSTRACT

Naphthoquinones are naturally occurring biologically active entities. Practical de novo syntheses of three naphthoquinones i.e. lawsone (**1**), lapachol (**2**), and β-lapachone (**3b**) have been achieved from commercially available starting materials. The conversion of lapachol (**2**) to β-lapachone (**3b**) was achieved through p-TSA/Iodine/BF$_3$-etherate mediated regioselective cyclisation. Further, 2-alkyl and 2-benzyllawsone derivatives have been prepared as possible anticancer agents. Four derivatives exhibited significant anticancer activity and the best analogue i.e. compound **21a** exhibited potential anticancer activity (IC$_{50}$ = 5.2 μM) against FaDu cell line. Compound **21a** induced apoptosis through activation of caspase pathway and exerted cell cycle arrest at S phase in FaDU cells. It also exhibited significant topoisomerase-II inhibition activity. Compound **21a** was found to be safe in Swiss albino mice up to 1000 mg/kg oral dose.

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1. Introduction

Naphthoquinones are common sub-structures in many natural products (Fig. 1).[1a–g] Naphthoquinones possess diverse pharmacological activities viz. anticancer,[2a–g] antimalarial,[3a,3b] antileishmania.[4a,4b] Lawsone (**1**), lapachol (**2**), and β-lapachone (**3b**) are biologically active important naphthoquinones. Lawsone (**1**) is the main dye component of Lawsonia Inermis L. (Family: Lythraceae, Henna plant, ∼1.7% in leaves) used mainly as cosmetic to colour hair, palm and skin.[5] It is used for the synthesis of some clinically important drugs like atovaquone, dichloroallyllawsone and lapachol. Lapachol (**2**) is a prenylated naphthoquinone isolated from Tabebuia avellannedeae,[6] Heterophragma adenophyllum[7] and Austroplenckia populnea.[8] It has been evaluated as cancer drug candidate through clinical trials. But, it was discontinued due to toxicity concerns.[9a,9b] β-Lapachone (**3b**) is a cyclised derivative of lapachol possessing potent anticancer activity through induction of DNA topoisomerase-II mediated DNA cleavage.[10a,10b] It induces caspase and p53 independent apoptotic pathways through NAD (P)H:quinone oxidoreductase 1 catalysed reduction.[11] It has been evaluated through six clinical trials, against pancreatic cancer,

head and neck cancer and advanced solid cancer in combination therapies.[12a,12b] One of its formulations is under phase II clinical trial under the codeARQ501 in combination with gemcitabine with hydroxypropyl-β-cyclodextrin (HPβCD)[13] for the treatment of pancreatic cancer. Being an important class of compounds, several partial and total syntheses of naphthoquinones have been done.[14–17] Previously, several partial syntheses of lapachol have been done using lawsone as starting substrate.[6,15] To the best of our knowledge, there is no total synthesis reported so far.

We report herein straight-forward total syntheses of lawsone (**1**), lapachol (**2**), and a regioselective synthesis of β-lapachone (**3b**). The conversion of lapachol to α/β lapachone has been modulated with various reagents and solvents. Further lawsone has been modified to several new 2-alkylated and 2-benzylated analogues as possible anticancer agents. The best analogue of the series has been evaluated for cell cycle, mechanistic studies and toxicity.

2. Results and discussion

2.1. Chemistry

2.1.1. Synthesis of lawsone (**1**)

The synthesis of **1** is outlined in Scheme 1. Firstly, 2-naphthol (**4**) was methylated with dimethylsulphate to afford 2-methoxy-
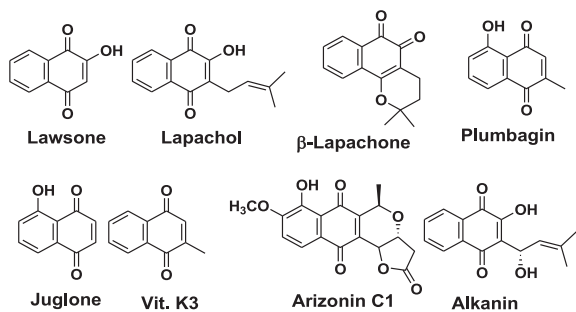
Fig. 1. Some representative natural naphthoquinones.

naphthalene (**5**) in 93% yield. Compound **5** on Vilsmeier-Haack formylation reaction using DMF-POCl$_3$ yielded the corresponding aldehyde **6** in 88% yield. Oxidation of aldehyde **6** with NBS in 95% DMF (aqueous) afforded naphthoquinone **7** in 77% yield.[18] Naphthophenone **7** on subsequent demethylation by anhyd. AlCl$_3$ yielded **1** in 91% yield. The overall yield of lawsone **1** was 57.34% in four synthetic steps.

In the recent past, lawsone was prepared from 1,4-quinone in three step synthesis.[14] In the final step 1,3,-dihydroxynaphthalene was converted to **1** using [bis (trifluoroacetoxy)-iodo]-benzene in acetonitrile-water. Thiele-Winter acetoxylation was the key reaction of the synthesis.

2.1.2. Synthesis of lapachol (2)

The retrosynthetic analysis of lapachol has been depicted in Scheme 2. 1,2,3,4-Tetrahydroxynaphthalene (**18**) is the key intermediate in this synthesis. Baeyer-Villiger oxidation can easily transform the formyl groups to the corresponding phenolic hydroxyl groups and formylation of naphthalene can be done through Vilsmeier reaction. Preparation of 1-chloro-2,3,-diformylnaphthalene (**9**) can be achieved from 2-tetralone in single step by Vilsmeier-Haack reaction. 1-Chloro group can be substituted by a methoxy group through nucleophilic substitution reaction using a suitable metal carbonate and methanol.[18]

As outlined in Scheme 3, β-tetralone (**8**) was used as starting substrate. On Vilsmeier-Haack formylation, tetralone **8** gave 1-chloro-2,3,-diformylnaphthalene (**9**) in excellent yield. Treatment of compound **9** with MeOH/Na$_2$CO$_3$ yielded the corresponding methyl ether **10**. Diformyl derivative **10**, on treatment with m-CPBA underwent Baeyer–Villiger oxidation to afford 1-methoxy-2-hydroxy-3-formylnaphthalene (**11**). Phenol **11** was methylated with dimethylsulphate to get 1,2-dimethoxy-3-formyl naphthalene (**12**). Further steps were similar to previous reactions, Baeyer-Villiger oxidation to afford phenol **13**, its methylation to get 1,2,3-trimethoxynaphthalene (**14**), subsequent formylation of **14** to give 1,2,3-trimethoxy-4-formylnaphthalene (**15**), m-CPBA
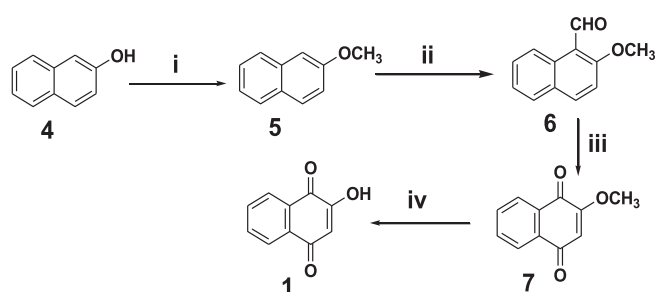
Scheme 1. Reaction conditions and reagents: (i) Me$_2$SO$_4$, K$_2$CO$_3$, dry acetone, 56 °C, 1 h, 93%; (ii) DMF-POCl$_3$, 0 °C-RT-120 °C, 4 h, 88%; (iii) NBS, DMF-H$_2$O (95:5), RT, 16 h, 77%; (iv) Anhyd. AlCl$_3$, dry DCM, 40 °C, 3 h, 91%.

treatment of **15** to afford the corresponding phenol **16**, followed by methylation of **16** to get 1,2,3,4-tetramethoxynaphthalene (**17**). Purification of **16** was difficult due to a complex reaction mixture, hence it was *in-situ* methylated to get **17**. Compound **17** was demethylated with BBr$_3$ to get directly naphthoquinone moiety i.e. 3-hydroxylawsone (**18**), which on treatment with isoprene in presence of oxalic acid afforded the final product **2**. Present strategy provides an efficient procedure for the synthesis of **2** in eleven simple steps to get **2** in 31.64% overall yield.

Previously, several partial syntheses of **2** have been done by several researchers (Scheme 4).[15a–f] Lawsone was used as starting substrate and appropriate chains at 3-position were attached by various methods.[14] But, all these methods produced **2** in poor yields (33–43%) applying drastic reaction conditions. However, recently Ferreira et al.[15f] used an aldehydic chain in ethanol-water and formic acid through condensation-reduction at 3-position of **1** to afford **2**. But, here high pressure reactor was used under drastic reaction conditions (200 °C, 3000 psi) which makes it a tedious process. However, the yield of the reaction was good (78%).

In another approach, Gates and Moesta[16] converted leucoisonaphthazarin to **2** using two different chains. Firstly, isoprene was used to give **2** in 3.2% yield, while the treatment with second chain dimethylvinylcarbinol afforded **2** in 5.32% yields. Both the processes were unsuitable due to poor yields. Ghera and Ben-David (1985) prepared lapachol from a prenylated malonate in 25–30% overall yield in four step synthesis.[19]

2.1.3. Regioselective synthesis of β-lapachone (3b)

Previously, lapachol was cyclised using mineral acids to synthesize β-lapachone. Hooker found that on treatment with HCl, **2** transforms to α-lapachone (**3a**) while with H$_2$SO$_4$ **2** produces **3b** in 60% yield.[17b] Singh et al.[17b] transformed **2** to a mixture of both regioisomers (**3a/3b**) using aqueous NaNO$_2$-acetic acid. Watson et al. did regioselective conversion of lapachol to β-lapachone using FeCl$_3$.[17c] In our experiments, as shown in Table 1, we used diverse reagents to transform lapachol to α/β-lapachone (Scheme 5). In case of p-TSA, BF$_3$.etherate, polyphosphoric acid and iodine, β-lapachone was the exclusive product, while, zinc chloride afforded α-lapachone predominantly. This conversion was unsuccessful with molecular sieves 4 Å, silica gel (100–200 mesh) and sodium hydride (see Scheme 5 and Table 1).

Solvent selection played an important role in achieving regioselectivity of this reaction. It was observed that DCM was the most suitable solvent with p-TSA, BF$_3$-Et$_2$O and I$_2$. When DCM was replaced with THF/MeOH or toluene, either reaction was unsuccessful or regioselectivity was lost.

2.1.4. Preparation of 2 and 3-alkylated lawsone derivatives

Further, ten new analogues of lawsone were prepared at 2- and 3-positions (Scheme 6). We tried various condensation reagents to achieve alkylated and benzylated derivatives. Interestingly, mono O-alkylation/benzylated and 2,3-dialkylated/dibenzylated analogues were obtained. Different bases were used like K$_2$CO$_3$, NaH and TEA and acetone/DMF were used as solvents. In case of ethyl bromoacetate in DMF and TEA at RT C-alkylated product was obtained exclusively in 64% yield.

2.2. Biological evaluation of 2-benzyl lawsone derivatives

2.2.1. Antiproliferative activity

All the analogues were evaluated for antiproliferative activity against five human cancer cell lines i.e. MCF-7 (breast adenocarcinoma), DU-145 (prostate carcinoma), DLD1 (colorectal adenocarcinoma), A549 (lung carcinoma) and FaDu (hypopharyngeal carcinoma) by Sulphorhodamine assay (Table 2).[20]
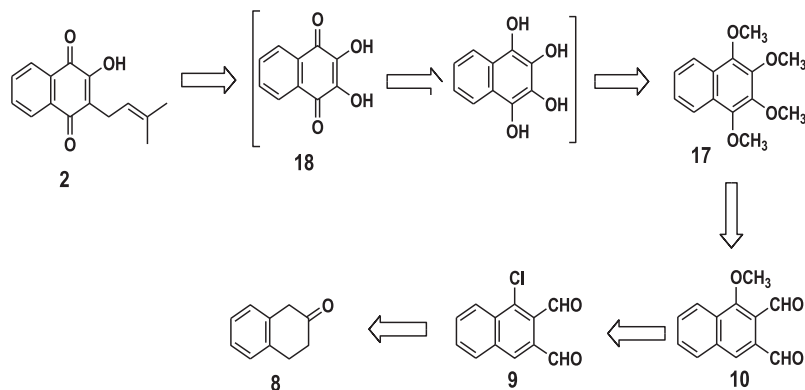
Scheme 2. Retrosynthetic analysis of lapachol (**2**).
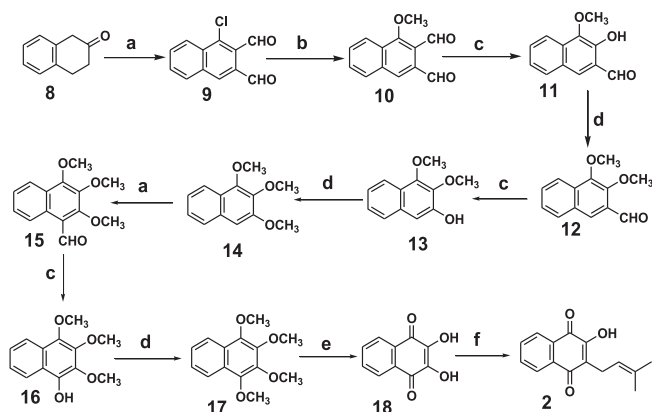
Scheme 3. Reaction conditions and reagents: (a) DMF-POCl₃, 0–30 °C, 1 h, for **14** to **15** after 30 min 120 °C; (b) Na₂CO₃, MeOH, 65 °C, 6 h; (c) *m*-CPBA, dichloromethane, Na₂HPO₄, 30 °C, 8 h; (d) Me₂SO₄, K₂CO₃, dry acetone, 30 °C, 1 h; (e) BBr₃, dry dichloromethane, −78 °C for 2 h, then 30 °C, 8 h; (f) Isoprene, oxalic acid, dioxane, 100 °C, 60 h.
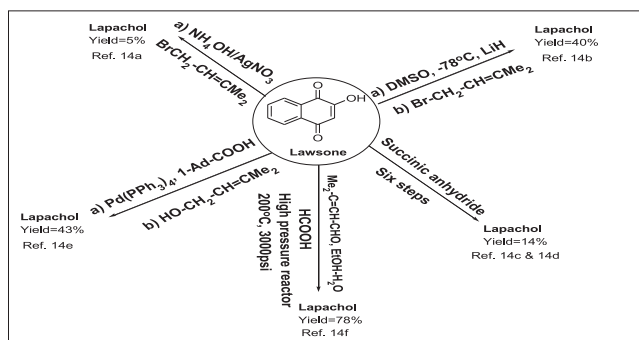
Scheme 4. Previous approaches to prepare lapachol from lawsone.

Four of the analogues (**19a**, **19b**, **21a** and **23a**) exhibited significant anticancer activity (IC$_{50}$ = 5.2–30 μM). Analogue **21a** exhibited good activity against all the five cell lines and potent activity against FaDu (IC$_{50}$ = 5.27 μM) and A549 (IC$_{50}$ = 6.83 μM) human cancer cell lines. All the four active analogues did not show cytotoxicity against healthy cell line HEK-293 up to 150 μM concentration. It was further evaluated for mechanistic and toxicity studies.

2.2.2. DNA Topoisomerase-II inhibition activity

Analogue **21a** exhibited significant topoisomerase-II inhibition activity (Fig. 2).[21] However, topo-II inhibition activity of compound **21a** was less than the activity of β-lapachone and etoposide. Topoisomerases (TOP-I and II) are important enzymes acting on the topology of DNA during its replication and transcription. Both of these regulate winding of DNA. TOP-I cuts one strand of DNA, while TOP-II cuts both strands of DNA double helix to relax it. Topoisomerases are important targets and their inhibitors are used extensively for treating human malignancies.[22] Naphthoquinones are potential topoisomerase inhibitors.[23,24] Several DNA targeting drugs like etoposide lead to topoisomerase-II mediated chromosome DNA breakage and death of cancer cell.[25] Previously, compound **21a** has been reported for various pharmacological activities; (a) antiplatelet activity by inhibiting arachidonic acid, collagen and platelet activativating factor, (b) antiinflammatory activity by neutrophil degranulation inhibition, and (c) antiallergic activity by mast cell degradanulation inhibition.[26]

2.2.3. Cell cycle analysis

In cell cycle analysis[27] compound **21a** induced S phase arrest in FaDu cells and at 10 μM concentration apoptosis was also observed (Fig. 3). Topo-II inhibitors generally induce S-phase arrest.[28] Cell cycle regulation ensures the fidelity of genomic replication and cell division.[29] But, uncontrolled proliferation is the peculiarity of cancer cells. Cancer cells typically acquire damage to genes that regulate their cell cycle.[30] Cell cycle arrest is prevention of normal progression of the cell cycle. The two major checkpoints G$_1$/S and G$_2$/M transitions allow the cells to control any modification in DNA content. Induction of cell cycle arrest in cancer cell lines constitutes one of the most prevalent strategies to stop or limit cancer spreading.[30,31]

2.2.4. Apoptosis induction through caspase pathway

Apoptosis is an important process in multicellular organisms but avoided by cancer cells. Poly-ADP ribose polymerase (PARP) is an enzyme involved in DNA repair when cells are exposed to environmental stress.[32] During apoptosis, PARP is cleaved and inactivated by caspases.[33] Therefore, cleavage of PARP is a well established marker for detection of apoptosis. Caspases are a family of cysteine-aspartic proteases, which are crucial mediators of apoptosis. Among these caspase-3 encoded as CAS3 gene is identified in numerous mammals. It further activates caspases 6, 7 and 9. Caspase-3 is necessary for its typical role in apoptosis, where it is responsible for chromatin condensation and DNA fragmentation.[34] The apoptosis was also induced by the inhibition of topoisomerase-II by compound **21a** at S-phase of cell cycle and subsequent interference in DNA synthesis. In Western blot analysis compound **21a** exerted PARP cleavage at 5 μM and 10 μM concentrations and thus induced apoptosis through caspase pathway (see Figs. 3 and 4).

Table 1
Conversion of lapachol to α/β-lapachone under various reaction conditions.

S. no.	Reagent	Solvent	Reaction condition	Reaction time (h)	% conv.	3a:3b
1.	$AlCl_3$	DCM	RT[a]	1	92	50:50
2.	P_2O_5	DCM	RT	2	89	20:80
3.	p-TSA	DCM	RT	1.5	84	0:100
4.	$BF_3.Et_2O$	DCM	RT	2	100	0:100
5.	PPA	Neat	80 °C	2	93	0:100
6.	I_2	DCM	RT	1	97	0:100
7.	$ZnCl_2$	DCM	42 °C	4	94	90:10
8.	NaH	DCM	RT/42 °C	4/2	0	No Rn
9.	Silica gel	DCM	RT/42 °C	4	0	No Rn
10.	Molecular sieves 4A°	DCM	RT/42 °C	4	0	No Rn

[a] RT = Room Temperature = 32–37 °C.
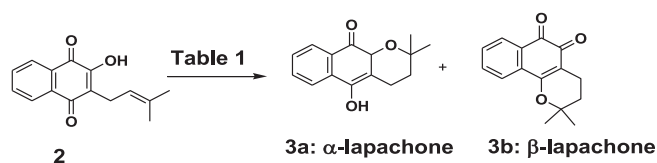
Scheme 5. Conversion of lapachol to α/β-lapachone.
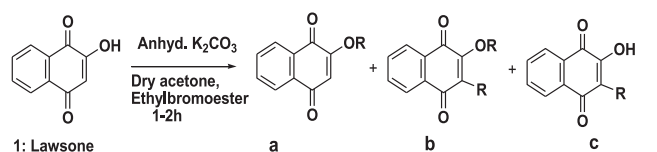
19a/b/c: R=-CH_2-$COOCH_2CH_3$; **20a:** R=-CH_2-CH=CH-$COOCH_2CH_3$;
21a/b: R=Benzyl; **22a:** R=3,5-Dimethoxybenzyl;
23a: R=3,4,5-Trimethoxybenzyl; **24b:** R=3,4-Dimethoxybenzyl;
25b: R=3,4-Methylenedioxybenzyl

Scheme 6. Alkylation and benzylation of lawsone.

2.2.5. Safety studies through acute oral toxicity

Acute oral toxicity describes the adverse effects of a compound on exposure for a short period of time to get an idea about the safety aspect of a bioactive compound. For safety evaluation[35] compound **21a** was given to Swiss albino mice at 5, 50, 300 and 1000 mg/kg dose once orally. No mortality was observed throughout the experimental period, there were non-significant changes in gait, posture and response of animals. Animals on gross pathological study did not show any significant changes in any of the organs studied, including their absolute and relative weight (Fig. 5). Blood and serum samples upon analysis showed non-significant changes in all the parameters studied like haemoglobin (Hb), RBC, WBC, differential leukocyte count (DLC), SGPT, ALP, creatinine, triglycerides, cholesterol, albumin, serum protein (Table 3 and Fig. 6). Therefore, the experiment showed that compound **21a** is well tolerated by the Swiss albino mice up to the dose level of 1000 mg/kg body weight as a single acute oral dose for 7 days. However,

sub-acute, chronic or sub-chronic experiments with the compound **21a** are needed to be carried out for any adverse effect on repeated exposure.[36]

3. Conclusion

Present study provided simple, straight-forward, and efficient processes for the syntheses of lawsone, lapachol, and β-lapachone in 31.64%, 57.35%, and 84% of overall yields, respectively. Regioselective synthesis of β-lapachone and modulation of reaction towards α-lapachone have been achieved. 2-Benzyllawsone exhibited potential anticancer activity by inducing apoptosis through caspase pathway and topoisomerase-II inhibition. It was safe up to 1000 mg/kg dose in Swiss-albino mice. The study encourages to optimize the identified lead molecule for better activity.

4. Experimental section

4.1. General

All the reagents were used as such without further purification. Starting substrates, *viz.* 2-naphthol was procured from Avra Synthesis, India, and 2-tetralone was purchased from Alfa Aesar India. Dry solvents were prepared as per standard methods and stored under molecular seives. Column chromatography was done on Teledyne 200i Flash chromatography in glass columns using 230–400 mesh silica gel and UV detector (254 nm and 365 nm) at flow rate of 16 mL/min. Melting points were taken in the open capillaries on E-Z Melt automated melting point apparatus (Stanford Research System, USA) and were uncorrected. NMR spectra were recorded on Bruker 300 MHz and 500 MHz spectrometer, chemical shifts are reported in δ scale (TMS = 0.0 ppm). Coupling constants (J value) are given in Hz, multiplicity is defined as s = singlet, d = doublet, t = triplet, m = multiplet. Electrospray ionisation mass were record on Shimadzu LC-MS system after dissolving compounds in methanol. High resolution mass were obtained on Agilent 6520Q-TOF using ESI. FT-IR spectra were recorded on Perkin–Elmer SpectrumBX.

Table 2
Antiproliferative activity of lawsone analogues.

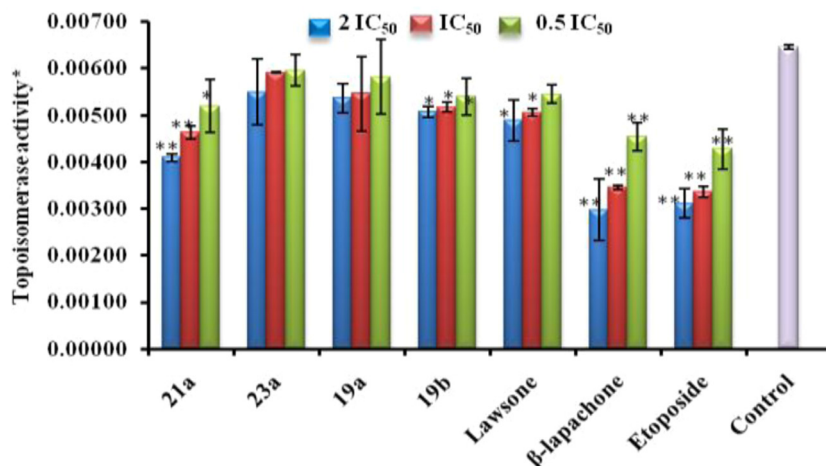| Compd. no.	Antiproliferative activity IC$_{50}$ in µM						% Inhibition DNA Topo-II at 2×IC$_{50}$
	MCF-7	DU-145	DLD1	A549	FaDu	HEK-293	
19a	>30	>30	>30	15.52 ± 1.28	19.73 ± 0.69	>150	16.8
19b	>30	>30	>30	21.06 ± 2.39	21.82 ± 1.76	>150	21.4
21a	13.87 ± 6.88	10.57 ± 1.83	16.22 ± 4.21	6.83 ± 2.12	5.27 ± 0.14	>150	36.6
23a	>30	25.24	>30	18.94 ± 3.65	16.53 ± 0.58	>150	14.7
ETP[a]	>30	10.64 ± 0.42	8.11 ± 0.23	>30	16.01 ± 1.12	7.51 ± 0.32	51.7
3b	–	–	–	–	–	25.31 ± 1.28	53.9

[a] ETP = etoposide.

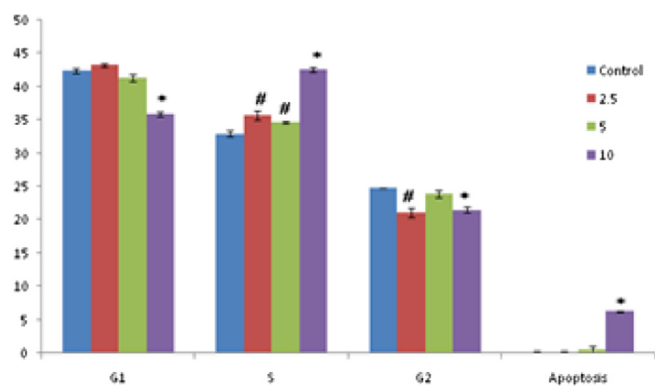**Fig. 2.** Topoisomerase-II inhibition by lawsone derivatives.

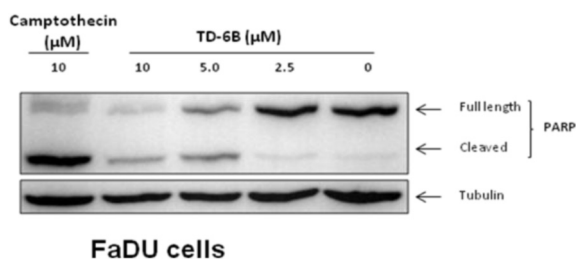**Fig. 3.** Effect of compound **21a** on different phases of cell cycle in FaDu cells.

Fig. 4. PARP cleavage by compound **21a** in FaDU cells.

4.2. Synthesis

4.2.1. General procedure for the synthesis of 5, 12, 14 and 17

4.2.1.1. 2-Methoxynaphthalene (5). To a solution of **4** (2 g, 13.89 mmol) in dry acetone (20 mL), anhydrous potassium carbonate (6 g, 43.48 mmol) and dimethylsulphate (1.5 mL, 15.83 mmol) were added and stirred at room temperature for an hour. The reaction mixture was filtered, washed with acetone and evaporated. The residue was taken in ethyl acetate (30 mL) and washed with water. The organic phase was dried with anhydrous sodium sulphate and concentrated *in-vacuo*. The residue was purified through flash chromatography column using hexane-chloroform to get pure methylated product **5** as white solid (2.04 g, 93%): mp 69–70 °C; IR (KBr) ν_{max} 3019, 1650, 1384, 1216, 1046; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 4.14 (s, 3H), 7.38–7.45 (m, 2H), 7.55–7.60 (t, J = 7.5 Hz, 1H), 7.66–7.71 (t, J = 7.5 Hz, 1H), 7.96–8.02 (m, 3H); ^{13}C NMR
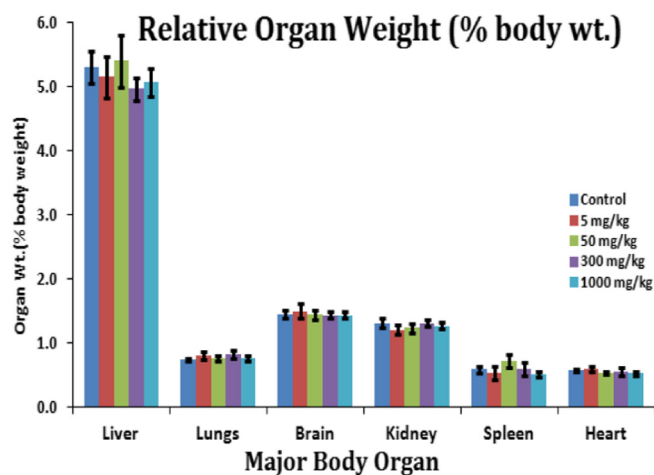

Fig. 5. Effect of compound **21a** as a single acute oral dose on absolute and relative organ weight in Swiss albino mice (Mean ± SE; n = 6).

(CDCl$_3$, 75 MHz) δ 55.7, 106.2, 119.1, 124.0, 126.8, 127.2, 128.1, 129.4, 129.8, 135.0, 158.1; Electrospray mass (MeOH) for C$_{11}$H$_{10}$O: 159 [M+H]$^+$.

4.2.1.2. 1,2,-Dimethoxy-3-formylnaphthalene (12). Yield 94%; yellow liquid; IR (Neat) ν_{max} 3020, 2399, 1731, 1637, 1384, 1215, 1068; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 4.03 (s, 3H), 4.06 (s, 3H), 7.42–7.47 (t, J = 7.5 Hz, 1H), 7.55–7.60 (t, J = 7.5 Hz, 1H), 7.88–7.90 (d, J = 6.0 Hz, 1H), 8.10–8.13 (d, J = 9.0 Hz, 1H), 8.13 (s, 1H), 10.48 (s, 1H); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 61.5, 62.4, 122.1, 126.2, 126.4, 129.1, 129.4, 130.3, 130.5, 132.9, 147.8, 149.6, 190.6; Electrospray mass (MeOH) 217 [M+H]$^+$, 239 [M+Na]$^+$; HRMS (ESI-TOF) m/z [M+H]$^+$calcd for C$_{13}$H$_{12}$O$_3$ 217.0865, found 217.0889.

4.2.1.3. 1,2,3-Trimethoxynaphthalene (14). Yield 92%, yellow liquid; IR (Neat) ν_{max} 3020, 1619, 1384, 1248, 1217; 3019, 1637, 1384, 1215; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 3.98–4.07 (m, 9H), 6.96 (s, 1H), 7.35–7.43 (m, 2H), 7.68–7.71 (d, J = 7.2 Hz, 1H), 8.06–8.08 (d, J = 7.2 Hz, 1H); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 56.2, 61.5, 61.8, 102.8, 122.0, 124.1, 124.8, 126.1, 126.8, 131.2, 141.3, 148.3, 153.4; Electrospray mass (MeOH) for C$_{13}$H$_{14}$O$_3$: 219.2 [M+H]$^+$ 0.241.1 [M+Na]$^+$, 257.2 [M+K]$^+$.

4.2.1.4. 1,2,3,4-Tetramethoxynaphthalene (17). Yield 96%, yellow liquid; IR (Neat) ν_{max} 3019, 1637, 1384, 1215, 1068; ^{1}H NMR

Table 3
Effect of compound **21a** as a single acute oral dose at 5, 50, 300 and 1000 mg/kg of body weight, hematological and serum biochemical parameters in Swiss albino mice (Mean ± SE; n = 6).

Parameters	Effect of compd **21a** on mg/kg body weight as a single oral dose in Swiss albino mice				
	Control	5 mg/kg	50 mg/kg	300 mg/kg	1000 mg/kg
Body weight (g)	27.73 ± 0.94	26.34 ± 1.55	26.76 ± 1.38	27.09 ± 0.69	26.48 ± 0.92
Haemoglobin (g/dL)	13.29 ± 0.46	12.65 ± 0.31	13.26 ± 0.35	13.42 ± 0.43	13.28 ± 0.23
RBC (million/mm^3)	9.07 ± 1.13	8.67 ± 1.18	8.54 ± 0.62	9.59 ± 0.36	8.89 ± 0.45
WBC (1000/mm^3)	8.64 ± 0.42	8.66 ± 0.42	8.38 ± 0.38	8.52 ± 0.23	8.70 ± 0.32
ALP (U/L)	292.13 ± 40.29	307.74 ± 45.51	304.24 ± 38.84	386.48 ± 25.80	385.91 ± 12.34
SGOT (U/L)	34.32 ± 3.78	38.30 ± 4.27	30.19 ± 1.81	33.87 ± 1.91	31.68 ± 2.88
SGPT (U/L)	23.06 ± 1.92	19.16 ± 2.07	18.02 ± 0.99	20.48 ± 1.72	25.00 ± 1.91
Albumin (g/dL)	2.49 ± 0.17	2.56 ± 0.18	2.70 ± 0.12	2.92 ± 0.11	2.88 ± 0.12
Creatinine (mg/dL)	0.09 ± 0.01	0.14 ± 0.02	0.16 ± 0.02	0.13 ± 0.01	0.16 ± 0.02
Triglycerides (mg/dL)	107.9 ± 10.70	130.9 ± 19.53	112.9 ± 14.61	119.3 ± 8.70	127.4 ± 11.19
Serum Protein (mg/mL)	2.91 ± 0.31	2.55 ± 0.32	2.92 ± 0.15	2.43 ± 0.17	2.95 ± 0.25
Cholesterol (mg/dL)	68.37 ± 4.42	65.28 ± 4.06	65.13 ± 3.59	45.62 ± 4.19	51.05 ± 4.27
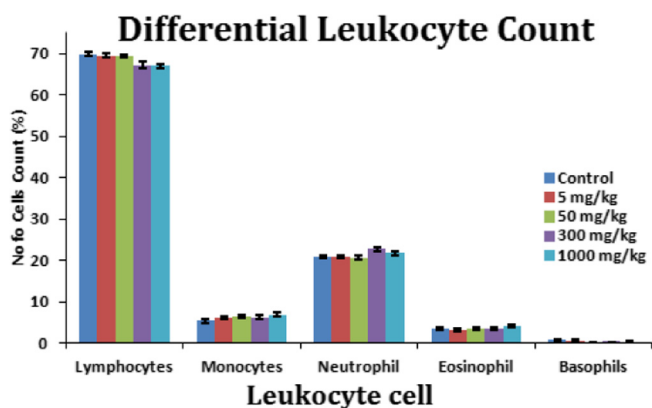
Fig. 6. Effect of compound **21a** as a single acute oral dose on differential leukocyte counts in Swiss albino mice (Mean ± SE; n = 6).

(CDCl$_3$, 300 MHz) δ 4.01 (s, 6H), 4.03 (s, 6H), 7.41–7.44 (m, 2H), 8.05–8.08 (m, 2H); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 61.7, 61.9, 121.9, 125.6, 126.0, 144.4, 144.8. Electrospray mass (MeOH) for C$_{14}$H$_{16}$O$_4$: 271 [M+Na]$^+$.

4.2.2. General procedure for the synthesis of 6, 9 and 15
4.2.2.1. 1-Formyl-2-methoxynaphthalene (6). A solution of **5** (1 g, 6.33 mmol) in dry DMF (5 mL) was cooled to 0 °C, and POCl$_3$ (2 mL, 21.43 mmol) was added drop-wise. After being stirred at 0 °C for an hour, the mixture was heated at 95 °C for 4 h. The reaction was quenched with ice-cold water, extracted with ethyl acetate (20 mL × 2) and washed with water. Organic phase was dried with anhydrous Na$_2$SO$_4$ and concentrated *in-vacuo* to get a residue. The residue was purified by flash column chromatography (ethyl acetate-hexane) to get **6** as creamy solid (1.04 g, 88%): mp 80–81 °C; IR (KBr) ν_{max} 3019, 2929, 1673, 1607, 1579, 1487, 1384; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 4.00 (s, 3H), 7.22–7.25 (t, J = 9.0 Hz, 1H), 7.39–7.43 (t, J = 6.0 Hz, 1H), 7.59–7.68 (m, 2H), 8.00–8.03 (d, J = 9.0 Hz, 1H), 9.27–9.30 (d, J = 9.0 Hz, 1H), 10.81 (s, 1H); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 56.9, 112.9, 116.9, 125.1, 125.3, 128.6, 128.9, 130.2, 131.9, 137.9, 164.3, 192.3. Electrospray mass (MeOH) for C$_{12}$H$_{10}$O$_3$: 187.1 [M+H]$^+$, 209.2 [M+Na]$^+$, 225.2 [M+K]$^+$.

4.2.2.2. 1-Chloro-2,3-diformylnaphthalene (9). Yield 91%; white solid: mp 69–71 °C; IR (KBr) ν_{max} 3381, 3021, 1731, 1619, 1381, 1246; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.51–7.55 (t, J = 15.0 Hz, 1H), 7.65–7.70 (t, J = 15.0 Hz, 1H), 7.86–7.89 (d, J = 9.0 Hz, 1H), 8.48 (s, 1H), 8.88–8.91 (d, J = 9.0 Hz, 1H), 10.56 (s, 1H), 10.84 (s, 1H); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 125.3, 128.4, 128.6, 129.8, 131.0, 131.6,

132.9, 133.9, 137.1, 140.2, 189.0, 192.1; Electrospray mass (MeOH) for C$_{12}$H$_7$ClO$_2$: 219.2 [M+H]$^+$, 241.1 [M+Na]$^+$.

4.2.2.3. 4-Formyl-1,2,3-trimethoxynaphthalene (15). Yield 91%, brown liquid; IR (Neat) ν_{max} 3019, 2926, 1621, 1384, 1215, 1069; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 3.97 (s, 3H), 4.09 (s, 3H), 4.24 (s, 3H), 7.44–7.46 (t, J = 3.9 Hz, 1H), 7.57–7.60 (t, J = 7.8 Hz, 1H), 8.13–8.16 (d, J = 8.1 Hz, 1H), 9.26–9.29 (d, J = 8.4 Hz, 1H), 10.71 (s, 1H). ^{13}C NMR (CDCl$_3$, 75 MHz) δ 61.8, 62.0, 63.4, 119.2, 122.1, 126.1, 126.2, 126.5, 128.5, 128.9, 142.1, 155.0, 162.5, 191.5; Electrospray mass (MeOH) 247.2 [M+H]$^+$ 0.269.1 [M+Na]$^+$, 285.2 [M+K]$^+$; HRMS (ESI-TOF) m/z [M+H]$^+$ calcd for C$_{14}$H$_{15}$O$_4$ 247.0970, found 247.0964.

4.2.3. General procedure for the synthesis of 11, 13 and 16
4.2.3.1. 3-Formyl-2-hydroxy-1-methoxynaphthalene (11). To a solution of **10** (1 g, 4.67 mmol) in dichloromethane (15 mL), saturated solution of Na$_2$HPO$_4$ (0.5 mL) and m-CPBA (1.5 g, 6.72 mmol, 77%) were added. The reaction mixture was stirred at 0 °C for 30 min and afterwards, at room temperature for 8 h. Solvent was evaporated and the residue was taken in ethyl acetate (25 mL) and washed with 5% NaHCO$_3$ solution (10 mL × 2). Organic phase was dried with anhydrous Na$_2$SO$_4$ and evaporated to get a residue. It was purified through flash column chromatography (hexane-ethyl acetate) to get **11** as yellow solid (957 mg, 90%): mp 88–89 °C; IR (KBr) ν_{max} 3398, 3019, 2926, 1654, 1603, 1384, 1318; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 3.99 (s, 3H), 6.53 (bs, 1H), 7.47–7.52 (t, J = 7.5 Hz, 1H), 7.57–7.62 (t, J = 7.5 Hz, 1H), 7.87 (s, 1H), 7.90–7.92 (d, J = 6.0 Hz, 1H), 8.19–8.22 (d, J = 8.4 Hz, 1H), 10.30 (s, 1H); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 63.8, 122.5, 125.9, 126.9, 128.0, 128.3, 128.8, 129.7, 130.6, 141.1, 144.8, 190.7: Electrospray mass (MeOH) for C$_{12}$H$_{10}$O$_3$: 203.2 [M+H]$^+$, 225.2 [M+Na]$^+$, 241.1 [M+K]$^+$.

4.2.3.2. 1,2-Dimethoxy-3-hydroxynaphthalene (13). Yield 93%, red liquid; IR (Neat) ν_{max} 3400, 3019, 1384, 1216, 1046; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 4.06 (s, 3H), 4.11 (s, 3H), 6.27 (bs, 1H), 7.16 (s, 1H), 7.36–7.41 (m, J = 7.5 Hz, 2H), 7.69–7.72 (d, J = 9.0 Hz, 1H), 8.07–8.10 (d, J = 9.0 Hz, 1H); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 61.5, 61.5, 105.8, 121.7, 123.9, 124.5, 126.2, 126.9, 131.6, 139.4, 146.9, 148.7; Electrospray mass (MeOH) for C$_{12}$H$_{12}$O$_3$: 205.2 [M+H]$^+$, 227.2 [M+Na]$^+$, 243.1 [M+K]$^+$.

4.2.3.3. 4-Hydroxy-1,2,3-trimethoxynaphthalene (16). Yield 89%, brown gummy liquid; IR (cm^{-1}) 3399, 3020, 2926, 1618, 1383, 1216; Electrospray mass (MeOH) for C$_{13}$H$_{14}$O$_4$: 273 [M+K]$^+$; Negative mode, 233 [M−H]$^-$.

*4.2.3.4. 2-Methoxynaphthalene-1,4-dione (**7**).* A solution of **6** (250 mg, 1.34 mmol) in DMF-H$_2$O (95:5, 12 mL), N-bromosuccinimide (620 mg, 3.5 mmol) was added and stirred at room temperature for 15 min. Afterwards, it was heated at 80 °C for 16 h. Water (10 mL) was added to it and extracted with ethyl acetate (15 mL × 2). The organic phase was dried with anhydrous Na$_2$SO$_4$ and concentrated *in vacuo*. The residue, thus obtained was purified by flash column chromatography (hexane-ethyl acetate) to get **7** as yellow solid (195 mg, 77%): mp 184–185 °C; IR (KBr) ν_{max} 3020, 2927, 1732, 1619, 1384, 1216, 1046; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 3.97 (s, 3H), 6.24 (s, 1H), 7.75–7.84 (m, *J* = 9.0 Hz, 2H), 8.09–8.20 (m, *J* = 5.5 Hz, 2H); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 56.8, 110.3, 126.5, 127.1, 131.4, 132.4, 133.7, 134.7, 160.8, 180.4, 185.2. Electrospray mass (MeOH) 189 [M+H]$^+$; HRMS (ESI-TOF) *m/z* [M+H]$^+$calcd for C$_{11}$H$_9$O$_3$ 189.0552, found 189.0547.

*4.2.3.5. 2-Hydroxynaphthalene-1,4-dione (**1**).* To a solution of **7** (100 mg, 0.53 mmol) in dry dichloromethane (10 mL), anhydrous aluminium chloride (200 mg, 1.5 mmol) was added and mixture was refluxed for 3 h. It was quenched with ice-cold water, acidified with HCl (5%, 3 mL), extracted with ethyl acetate, washed with water and dried with anhydrous Na$_2$SO$_4$. The organic phase was purified by a filter column of silica gel (60–120 mesh) eluting with ethyl acetate-hexane to get **1** as yellow solid (84 mg, 91%): mp 194–195 °C (192 °C)[17b]; IR (KBr) ν_{max} 3399,3020, 1734, 1654, 1384, 1216, 1068; ^{1}H NMR (CD$_3$COCD$_3$ + CD$_3$OD, 300 MHz) δ 6.19 (s, 1H), 7.75–7.83 (m, *J* = 9.0 Hz, 2H), 7.98–8.01 (d, *J* = 9.0 Hz, 2H); ^{13}C NMR (CD$_3$COCD$_3$ + CD$_3$OD, 75 MHz) δ 111.1, 126.0, 126.3, 130.9, 132.9, 133.4, 134.9, 159.1, 181.8, 185.2. Electrospray mass (MeOH) 213 [M+K]$^+$; Neg. Mode: 173 [M–H]$^-$; HRMS (ESI-TOF) *m/z* [M+H]$^+$calcd. for C$_{10}$H$_7$O$_3$ 175.0395, found 175.0390.

*4.2.3.6. 2,3-Diformyl-1-methoxynaphthalene (**10**).* To a stirred solution of **9** (200 mg, 0.92 mmol) in methanol (10 mL), anhydrous Na$_2$CO$_3$ (1 g, 9.43 mmol) was added and reaction mixture was refluxed for 6 h. Reaction mixture was filtered and filtrate was evaporated. The residue was taken in ethyl acetate (25 mL) and washed with water (10 mL). The organic phase was dried with anhydrous Na$_2$SO$_4$ and concentrated. The residue was purified by flash column chromatography (hexane-ethyl acetate) to get **10** as solid.

10: Yield: 174 mg (89%), white solid, mp 120–121 °C; IR (KBr) ν_{max} 3022, 1681, 1619, 1585, 1503, 1216, 1104; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 4.09 (s, 3H, OCH$_3$), 7.52–7.57 (t, 1H, aromatic, *J* = 7.5 Hz), 7.70–7.75 (t, 1H, *J* = 7.5 Hz), 7.92–7.94 (d, 1H, aromatic, *J* = 6.0 Hz), 8.59 (s, 1H), 9.17–9.20 (d, 1H, *J* = 6.0 Hz), 10.43 (s, 1H, 2-CHO), 10.81 (s, 1H, 3-CHO), ^{13}C NMR (CDCl$_3$, 75 MHz) δ 67.4, 123.4, 125.8, 127.4, 128.1, 130.2, 130.8, 132.7, 134.3, 139.4, 166.0, 188.9, 191.7; Electrospray mass (MeOH) 215.2 [M+H]$^+$, 237.1 [M+Na]$^+$, 253.2[M+K]$^+$. ESI-HRMS: 215.0703 for C$_{13}$H$_{10}$O$_3$, calcd 215.0708.

*4.2.4. Synthesis of 2,3-dihydroxynaphthalene-1,4-dione (**18**)*

A solution of **17** (200 mg, 0.81 mmol) in dry dichloromethane was cooled to –78 °C using acetone bath (Liq. N$_2$). After two hours, it was gradually allowed to stay at room temperature for 8 h. The reaction was quenched with dil. HCl (5%, 3 mL) and organic solvent was concentrated *in vacuo*. The residue was taken in ethyl acetate (20 mL) and washed with water. The organic phase was dried with anhydrous Na$_2$SO$_4$ and evaporated. The residue was purified by flash column chromatography (MeOH-CHCl$_3$) to get **18** as solid.

18: Yield: 137 mg (89%), brown colour solid, mp 265–266; IR (KBr) ν_{max} 3391, 2926, 1684, 1614, 1383, 1216; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.67–7.70 (d, *J* = 7.5 Hz, 2H), 7.84–7.87 (d, *J* = 8.1 Hz, 2H), ^{13}C NMR (CDCl$_3$, 75 MHz) δ m126.1, 130.7, 134.4, 141.1, 181.6; HRMS (ESI-TOF) *m/z* [M+H]$^+$calcd. for C$_{10}$H$_7$O$_4$ 191.0344, found 191.0341.

*4.2.5. Synthesis of 2-Hydroxy-3-(3-methylbut-2-enyl]-naphthalene-1,4-dione (**2**)*

To a stirred solution of **18** (100 mg, 0.53 mmol) in dry 1,4-dioxane (10 mL), anhydrous oxalic acid (100 mg, 0.79 mmol) was added. To this, isoprene (0.4 mL, 272 mg, 4.0 mmol) was added and the reaction mixture was refluxed at 100 °C for 60 h. Reaction mixture was filtered and filtrate was evaporated to get a residue. The residue was taken in ethyl acetate (30 mL) and washed with water (20 mL). Organic phase was dried with anhydrous Na$_2$SO$_4$ and concentrated *in vacuo*. The residue was purified by flash column chromatography to get compound **2** as yellow coloured solid.

Yield 99 mg (78%): mp 140–141 °C [139–140 °C][37]; IR (KBr) ν_{max} 3019, 2927, 1732, 1603, 1384, 1215; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 1.68 (s, 3H), 1.78 (s, 3H), 3.29–3.31 (d, *J* = 7.2 Hz, 2H), 5.18–5.23 (t, *J* = 7.1 Hz, 1H), 7.70–7.75 (m, 2H), 8.04–8.09 (m, 2H); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 23.0, 26.1, 30.0, 120.0, 123.9, 126.4, 127.1, 129.8, 133.2, 133.3, 134.1, 135.2, 153.1, 182.0, 184.9; Electrospray mass (MeOH) 241 [M–H]$^-$, HRMS (ESI-TOF) *m/z* [M+H]$^+$calcd for C$_{15}$H$_{15}$O$_3$ 243.1021, found 243.1020.

*4.2.6. Synthesis of α-lapachone (**3a**)*

To a stirred solution of **2** (100 mg, 0.29 mmol) in dry dichloromethane (10 mL), anhydrous zinc chloride (80 mg, 0.59 mmol) was added and reaction mixture was refluxed for 4 h. The reaction mixture was filtered and the filtrate was evaporated. Residue thus obtained was taken in ethyl acetate (25 mL) and washed with water. The organic phase was dried with anhydrous Na$_2$SO$_4$ and evaporated *in vacuo* to get a residue which on purification through a filter column of silica gel (hexane-ethyl acetate) afforded **3a** as light yellow coloured solid.

Yield 84 mg (90%); mp 119–120 [117–118][17b];^{1}H NMR (CDCl$_3$, 500 MHz) δ 1.46 (s, 6H, 2×CH$_3$), 1.83–1.86 (t, 2H, CH$_2$, *J* = 6.5 Hz), 2.63–2.66 (t, 2H, CH$_2$, *J* = 6.5 Hz), 7.68–7.72 (m, 2H, Aromatic), 8.09–8.11 (t, 2H, Aromatic, *J* = 4 Hz). ^{13}C NMR (CDCl$_3$, 125 MHz) δ 16.75, 26.52, 26.52, 31.46, 78.15, 120.16, 125.98, 126.33, 131.21, 132.12, 132.92, 133.85, 154.65, 180.01, 184.40; Electrospray mass (MeOH) 243.2 [M+H]$^+$, 265.1 [M+Na]$^+$, 281.4 [M+K]$^+$; Negative mode 241.2 [M–H]$^-$; HRMS (ESI-TOF) *m/z* [M+H]$^+$calcd for C$_{15}$H$_{15}$O$_3$ 243.1021, found 243.1025, *m/z* [M+Na]$^+$calcd for C$_{15}$H$_{14}$O$_3$Na 265.0840, found 265.0846.

*4.2.7. Synthesis of β-lapachone (**3b**)*

To a stirred solution of **2** (70 mg, 0.29 mmol) in dry dichloromethane (10 mL), p-toluenesulfonic acid (70 mg, 0.41 mmol) was added and further stirred at room temperature for 2 h. The reaction mixture was filtered and the filtrate was evaporated. Residue was taken in ethyl acetate (25 mL) and washed with water. Organic phase was dried with anhydrous Na$_2$SO$_4$ and concentrated *in vacuo*. The residue was purified through a filter column of silica gel (hexane-ethyl acetate) to get **3** as orange coloured solid.

Yield 59 mg (84%); mp 151–152.5 °C [154–55 °C][17b]; IR (KBr) ν_{max} 3399, 3019, 1732, 1654, 1384, 1215, 1047; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 1.22 (s, 3H), 1.25 (s, 3H), 1.80–1.85 (t, *J* = 6.5 Hz, 2H), 2.51–2.56 (t, *J* = 6.5 Hz, 2H), 7.44–7.49 (t, *J* = 7.5 Hz, 1H), 7.59–7.63 (t, *J* = 7.1 Hz, 1H), 7.77–7.79 (d, *J* = 7.5 Hz, 1H), 8.00–8.02 (d, *J* = 7.5 Hz, 1H). ^{13}C NMR (CDCl$_3$, 75 MHz) δ 16.5, 27.1, 27.1, 32.0, 79.7, 113.1, 124.4, 128.9, 130.5, 131.0, 133.0, 135.2, 162.4, 178.9, 180.2; Electrospray mass (MeOH) 243 [M+H]$^+$, 265 [M+Na]$^+$, 281 [M+K]$^+$; Negative mode 241 [M–H]$^-$; HRMS (ESI-TOF) *m/z* [M+H]$^+$calcd for C$_{15}$H$_{15}$O$_3$ 243.1021, found 243.1020; *m/z* [M+Na]$^+$calcd for C$_{15}$H$_{14}$O$_3$Na 265.0840, found 265.0837.

*4.2.8. General procedure for the synthesis of O-alkylated and C-alkylated derivatives of lawsone (**19a–25b**)*

Lawsone (250 mg, 1.43 mmol) was stirred in dry acetone (10 mL). To this anhydrous potassium carbonate (1 g, 7.24 mmol)

and bromo reagent (ethyl bromo esters/benzyl bromides, 2 mmol) were added and refluxed for 1–2 h. It was filtered and solvent was evaporated. The residue was taken in ethyl acetate, washed with water and dried over anhydrous sodium sulphate. The organic layer was evaporated and residue was purified through silica gel column to get *O*-alkylated and *C*-alkylated products.

The reaction also proceeds with triethylamine/DMF to get exclusive product **19b** in 63% yield.

4.2.8.1. Ethyl-2-(1,4-dihydro-1,4-dioxonaphthalen-2-yloxy)-acetate (19a). Yield 86%; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 1.28 (t, 3H, CH$_3$, J = 7.1 Hz), 4.25 (q, 2H, OCH$_2$, J = 7 Hz), 4.70 (s, 2H, OCH$_2$), 6.03 (s, 1H, 3-CH), 7.72 (m, 2H, 2×CH aromatic), 8.11 (m, 2H, 2×CH, aromatic); ^{13}C NMR (CDCl$_3$, 75 MHz) δ14.49, 62.41, 65.76, 111.45, 126.56, 127.15, 131.44, 132.21, 133.91, 134.74, 158.97, 166.64, 179.86, 184.98; Electrospray mass (MeOH) for C$_{14}$H$_{12}$O$_5$; 261 [M+H]$^+$; 283 [M+Na]$^+$; 299 [M+K]$^+$; Neg. Mode: 259 [M−H]$^-$.

4.2.8.2. Ethyl-3-(1,4-dihydro-1,4-dioxonaphthalen-2-ethoxycarbonyl methoxy-3-yl)-acetate (19b). Yield = 63%; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 0.87 (m, 6H, 2×CH$_3$), 3.74 (s, 2H, 3-CH$_2$), 4.10–4.24 (dq, 4H, 2×OCH$_2$), 5.08 (s, 2H, OCH$_2$), 7.70 (bs, 2H, 2×CH aromatic), 8.06 (m, 2H, 2×CH aromatic); ^{13}C NMR (CDCl$_3$, 75 MHz) δ14.52, 14.52, 23.06, 30.07, 61.46, 61.80, 114.44, 126.81, 127.66, 131.70, 132.07, 133.79, 134.55, 156.50, 169.11, 170.40, 181.96, 184.58; Electrospray mass (MeOH) for C$_{18}$H$_{18}$O$_7$; 347 [M+H]$^+$.

4.2.8.3. Ethyl-4-(1,4-dihydro-1,4-dioxonaphthalen-2-yloxy)-but-2-enoate (20a). Yield 91%; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 1.28 (t, 3H, CH$_3$), 4.14 (q, 2H, OCH$_3$), 4.32 and 4.74 (bs, 1H, OCH$_2$), 6.12–6.23 (m, 1H, CH=), 6.18 (s, 1H, 3-CH), 7.04 (d, 1H,=CH, J = 18.5 Hz), 7.75 (bs, 2H, 2×CH aromatic), 8.10 (m, 2H, 2×CH aromatic); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 14.59, 62.19, 67.79, 111.38, 120.58, 124.20, 126.62, 127.13, 133.26, 134.78, 139.31, 147.28, 159.13, 166.87, 180.05, 185.17; Electrospray mass (MeOH) for C$_{16}$H$_{14}$O$_5$; 309 [M+Na]$^+$; 325 [M+K]$^+$.

4.2.8.4. 2-(Benzyloxy)-naphthalene-1,4-dione (21a). Yield 68%; m.p. =183–185 °C; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 5.15 (bs,2H, OCH$_2$), 6.25 (bs (1H, 3-CH), 7.43 (bs, 5H, CH of phenyl ring), 7.74 (bs (2H, CH aromatic), 8.13 (bs, 2H, CH aromatic); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 71.49, 111.57, 126.52, 127.05, 127.35, 127.35, 127.99, 127.99, 128.93, 129.14, 131.59, 132.36, 133.71, 134.61, 159.73, 180.38, 185.28; Electrospray mass (MeOH) 287 [M+Na]$^+$; 303 [M+K]$^+$; Neg. Mode: 263 [M−H]$^-$; HRMS (ESI-TOF) m/z [M+H]$^+$calcd for C$_{17}$H$_{12}$O$_3$ 265.0864, found 265.0844.

4.2.8.5. 2-Benzyloxy-3-benzyl-naphthalene-1,4-dione (21b). Yield 27%; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 3.71 (s, 2H, CH$_2$), 5.29 (s, 2H, OCH$_2$), 7.01–7.39 (m, 10H, CH of both phenyl rings), 7.43 (bd, 2H, CH aromatic), 7.92 (bd, 2H, CH aromatic); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 29.89, 75.62, 126.58, 126.72, 126.75, 127.23, 128.90, 128.97, 129.09, 129.43, 129.66, 129.74, 129.89, 130.17, 131.96, 132.31, 133.31, 133.70, 134.28, 137.19, 139.51, 157.23, 182.32, 185.42; Electrospray mass (MeOH) for C$_{24}$H$_{18}$O$_3$; 355 [M+H]$^+$.

4.2.8.6. 2-(3′,5′-Dimethoxybenzyloxy)-naphthalene-1,4-dione (22a). Yield 87%; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 3.84 (s, 6H, 2×OCH$_3$), 5.08 (s, 2H, OCH$_2$), 6.20 (s, 1H, 3-CH), 6.57 (s, 2H, CH, aromatic of benzylic ring), 7.76 (m, 2H, CH, aromatic), 8.14 (m, 2H, CH aromatic); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 55.72, 55.80, 71.44, 100.06, 100.94, 105.67, 105.67, 111.71, 126.54, 127.05, 131.57, 132.37, 133.71, 134.62, 136.85, 159.65, 161.64, 180.40, 185.27; Electrospray mass (MeOH) for C$_{19}$H$_{16}$O$_5$; 325 [M+H]$^+$; Neg. Mode: 323 [M−H]$^-$.

4.2.8.7. 2-(3′,4′,5′-Trimethoxybenzyloxy)-naphthalene-1,4-dione (23a). Yield 89%; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 3.72 (s, 3H, OCH$_3$), 3.85 (s, 6H, 2×OCH$_3$), 4.94 (s, 2H, OCH$_2$), 6.22 (s, 1H, 3-CH), 6.64 (s, 2H, CH aromatic of benzyl ring), 7.72 (bs, 2H, CH aromatic), 8.07 (m, 2H, CH aromatic); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 56.39, 56.50, 56.54, 71.78, 105.27, 105.76, 114.45, 126.80, 127.06, 130.08, 131.53, 132.35, 133.77, 134.41, 134.69, 153.81, 154.04, 159.66, 180.42, 185.27; Electrospray mass (MeOH) for C$_{20}$H$_{18}$O$_6$; 377 [M+Na]$^+$; Neg. Mode: 353 [M−H]$^-$.

4.2.8.8. 2-(3′,4′-dimethoxyBenzyloxy)-3-(3′,4′-dimethoxybenzyl)-naphthalene-1,4-dione (24b). Yield = 48%; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 3.83 (s, 6H, 2×OCH$_3$), 3.92 (s, 6H, 2×OCH$_3$), 3.99 (s, 2H, CH$_2$), 5.38 (s, 2H, OCH$_2$), 6.26 (s, 1H, CH, aromatic), 6.37 (s, 1H, CH, aromatic), 6.45 (s, 2H, CH, aromatic), 6.58 (s, 2H, CH, aromatic), 7.68 (m, 2H, CH, aromatic), 8.10 (m, 2H, CH, aromatic); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 30.11, 55.56, 55.56, 55.71, 55.71, 75.41, 98.71, 100.84, 106.31, 107.54, 107.54, 107.68, 107.68, 126.56, 126.77, 131.89, 132.26, 133.71, 134.29, 139.34, 141.57, 157.23, 161.10, 161.10, 161.36, 161.36, 182.26, 185.39; Electrospray mass (MeOH) for C$_{28}$H$_{26}$O$_7$; 513 [M+K]$^+$; Neg. Mode: 473 [M−H]$^-$.

4.2.8.9. 2-(3′,4′-methylenedioxybenzyloxy)-3-(3′,4′-methylenedioxy-benzyl)-naphthalene-1,4-dione (25b). Yield 54%; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 3.34 (bs, 2H, CH$_2$), 4.14 (bs, 2H, OCH$_2$), 5.11 (s, 2H, O-CH$_2$-O), 5.99 (s, 2H, O-CH$_2$-O), 6.72–8.21 (m, 10H, CH aromatic); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 29.10, 71.01, 97.68, 101.20, 101.62, 108.34, 108.53, 108.70, 121.36, 121.45, 122.01, 122.63, 124.30, 127.98, 128.74, 130.77, 130.85, 146.77, 146.95, 148.00, 148.43, 152.99, 161.34, 161.86, 181.48, 185.23; Electrospray mass (MeOH) for C$_{26}$H$_{18}$O$_7$; 443 [M+H]$^+$.

4.3. Biological evaluation

4.3.1. Cell culture

Human cancer cell lines, MCF-7 (Breast adenocarcinoma), DU145 (Prostate carcinoma), DLD1 (colorectal adenocarcinoma), A549 (lung carcinoma), FaDU (Hypopharyngeal carcinoma) and HEK-293 (human embryo kidney cells) were originally obtained from American type of cell culture collection (ATCC), USA and cultured at 37 °C in DMEM supplemented with 10% FBS and Ab-Am (antibiotic-antimitotic) solution in a CO$_2$ incubator (New Brunswick/Eppendorf, Germany) under 5% CO$_2$ and 95% relative humidity. Etoposide was used as standard anticancer drug (positive control) for cytotoxicity.

4.3.2. Cytotoxicity evaluation

Cytotoxicity of lawsone derivatives was evaluated by Sulphorhodamine B assay.[20] Briefly, 10^4 cells/well were incubated overnight in 96-well culture plates at 37 °C in 5% CO$_2$. Next day, at 80% confluency, serial dilutions of test compound were added to the wells. Untreated cells served as control. After 48 h, cells were fixed with ice-cold 50% (w/v) trichloroacetic acid (100 μL/well) for 1 h at 4 °C. Cells were then stained with SRB dye (0.4% w/v in 1% acetic acid, 50 μL/well), washed and air-dried. Bound dye was solubilised with 10 mM Tris base (150 μL/well) and absorbance was read at 540 nm on a plate reader (Biotek, USA). The cytotoxic effect was calculated as:

$$\% \text{ inhibition in cell growth} = [1 - (\text{Absorbance of treated cells}/\text{Absorbance of untreated cells}) \times 100$$

Determination of 50% inhibitory concentration (IC$_{50}$) was based on dose-response curves.

4.3.3. Cell cycle analysis

The effect of most potent compound **21a** on FaDU cell division cycle was assessed by flow cytometry with PI-stained cellular DNA.[27] In brief, 4×10^5 cells per well were seeded in 6-well culture plate and grown overnight at 37 °C in 5% CO$_2$. After treating with compound **21a** for different time points, cells were harvested by trypsinization and fixed with ice-cold 70% ethanol for 30 min at 4 °C. The pellets were washed with PBS and re-suspended in a solution containing propidium iodide (20 mg/mL), TritonX100 (0.1%) and RNase (1 mg/mL) in PBS. After distribution of cells in different phases of cell cycle, "Cell Quest" software was used for calculations.

4.3.4. Western blot experiment

Compound **21a** treated (2.5 μM, 5.0 μM and 10 μM) FaDU cells were lysed (30 min, in ice) with cold M-PER (mammalian protein extraction reagent) supplemented with protease inhibitor cocktail. Camptothecin was taken as positive control at 10 μM concentration. Equal amount of proteins (tubulin, 25 μg) extracted from the cells treated at different time intervals were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred electrophoretically onto PVDF membranes. The membranes were blocked with 5% nonfat dry milk powder dissolved in Tris-buffer saline (TBS; 20 mM Tris-HCl, pH 7.6, 137 mM NaCl) containing 0.1% Tween20 (TBS-T) for an hour at RT (30 °C) and subsequently incubated overnight with anti-PARP antibody (cat# 9542, Cell Signalling Technology, USA) at 4 °C. After 3 washes with TBS-T, the membranes were incubated with HRP conjugated anti-rabbit antibody for 1 h at RT and washed thrice with TBS-T. Proteins were detected with an enhanced chemiluminescence (ECL) reagent and visualized by a chemiluminescence detector (Bio-Rad Laboratories, USA). The densitometry analysis of blots was done by using Bio-Rad Image Lab 4.0 software.

4.3.5. Topoisomerase-II inhibition assay

Assay was carried out as per the protocol described in Topoisomerase-II ELISA kit from Cloud-Clone Corp., USA (Catalog no. SEA792Hu).[21] Briefly, this assay employs an antibody specific for human topoisomerase-II coated on 96-well plate. Standards and samples (100 μL) were pipetted into the designated well as stated in the manual 100 μL of biotin-conjugated antibody specific to topoisomerase-II was added to the sample (cell lysate prepared after giving 24 h treatment in FaDu cell line) followed by washing with wash buffer (300 μL) which removed unbound biotinylated antibody. 100 μL of avidin conjugated horseradish peroxidase (HRP) was added into the wells and washed again. Finally, 90 μL of tetramethylbenzidine (TMB) substrate solution was added to the wells for colour development in proportion to the amount of bound topoisomerase-II. The stop solution (50 μL) changed the colour from blue to yellow, and the intensity of the colour was measured at 450 nm immediately. All samples were assayed in duplicate. Etoposide (1 mg/mL) was used as the standard inhibitor of topoisomerase-II.

The concentration of topoisomerase-II (TOP2) in the samples was determined from the standard curve and the total protein content was estimated). Activity of topoisomerase-II was expressed in terms of ng of topoisomerase per mg of total protein. Percent change/difference was also calculated for the ease of understanding.

4.3.6. Safety evaluation

In-vivo acute oral toxicity assay was performed by following the IAEC (Institutional Animal Ethical Committee) approved protocols as per our reported method.[35] In view of potent anti-cancer activity of compound **21a** in *in vitro* model, its acute oral toxicity was evaluated in Swiss albino mice for its safety aspects as an investigational drug. 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 (n = 6) weighing between 20 and 25 g. 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 I was kept as control and animals of Groups II, III, IV and V were test groups. The animals were acclimatized for 7 days in the experimental environment prior to the actual experimentation. Compound **21a,** suspended in carboxymethylcellulose (CMC, 0.7%) was given at 5, 50, 300 and 1000 mg/kg body weight to animals of Groups II, III, IV and V 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 compound **21a** and thereafter 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, changes in gait, posture and response to handling were also recorded. 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. The samples were analysed for total RBC, WBC, differential leucocytes count, haemoglobin percentage and biochemical parameters like ALP, SGPT, SGOT, total cholesterol, triglycerides, creatinine, bilirubin, serum protein and tissue protein activity. The animals were then sacrificed and were necropsed for any gross pathological changes. Weights of vital organs (liver, heart, kidney, etc.) were also recorded.

4.3.7. Statistical analysis

Statistical analysis for cytotoxicity was carried out in Microsoft Excel. The ANOVA followed by turkeys multiple comparison test was used to assess the statistical significance of vehicle verses treatment groups. Results are presented as the means ± SE. Differences with a P value <0.05 were considered significant. For topo-II inhibition assay, statistical analysis was done through Dunnett test and a *p* value of less than 0.05 was considered significance.

A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2016.12.043.

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