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Research paper

Synthesis of a series of novel dihydroartemisinin monomers and dimers containing chalcone as a linker and their anticancer activity

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ABSTRACT

A new series of monomer and dimer derivatives of dihydroartemisinin (DHA) containing substituted chalcones as a linker were synthesized and investigated for their cytotoxicity in human cancer cell lines HL-60 (leukemia), Mia PaCa-2 (pancreatic cancer), PC-3 (prostate cancer), LS180 (colon cancer) and HEPG2 (hepatocellular carcinoma). Some of these derivatives have greater antiproliferative and cytotoxic effects in tested cell lines than parent compound DHA. The structures of the all compounds were confirmed by IR, ^{1}H NMR and mass spectral data. Among the new derivatives, compounds **8**, **14**, **15**, **20** and **24** were found to be more active than parent DHA against tested human cancer cell lines. DHA derivatives were found to be most active in human leukemia cell lines with compounds **8**, **14**, **15**, **20** and **24** showed IC_{50} values less than 1 μM for 48 h whereas DHA has IC_{50} value of 2 μM at same time period. The most potent compounds **8** with $IC_{50} = 0.3$ μM (at par with doxorubicin ($IC_{50} = 0.3$ μM)) and **15** with $IC_{50} = 0.4$ μM, of the series, six and three times active than DHA (with $IC_{50} = 2$ μM) respectively were selected for further mechanistic work in human leukemia HL-60 cells.

1. Introduction

Artemisinin (**1**), isolated from *Artemisia annua* L. [1] contains a 1, 2, 4-trioxane moiety (Fig. 1). Artemisinin and its derivatives dihydroartemisinin (DHA, **2**), artemether (**3**), arteether (**4**), and artesunate (**5**) have been developed as a new type of antimalarial drugs [2a]. Recently we have synthesized twenty five ether and ester derivatives having significant antimalarial activity [2b]. In addition to their antiparasitic properties, it is of considerable interest to note that artemisinin derivatives are cytotoxic towards cancer cell lines *in vitro* [3]. Interestingly, the recent discovery of artemisinins as anticancer agents against various cancer cell lines have evoked many interests on this class of compounds [4-21a]. Artemisinin (**1**) and its derivatives are significantly cytotoxic towards murine lymphocytic leukemia (P-388), human lung carcinoma (A-549), human colon adenocarcinoma (HT-29) and other tumour cell lines [21b]. However, one of its derivatives appeared to be more cytotoxic than artemisinin (**1**) towards Ehrlich ascites tumour (EAT) cells [21c,21d].

Artemisinin dimers are obtained by joining two artemisinin molecules without destroying their endoperoxide bridge. Endoperoxide bridge in artemisinin is the key factor for its outstanding medicinal value. Due to sensitive nature of endoperoxide ring of artemsisinin majority of artemisinin analogues were synthesized via chemical modification of artemisinin at its C-10/C-13 position [22,23]. Beekman et al. synthesized C-10 acetal dimers where two artemisinin units are connected through an ether-linkage possessing good anticancer activity [24]. Posner et al. synthesized a series of C-10 acetal artemisinin dimers linked through a polyethylene glycol or carbon chain link or disulfide linker, with varying length and flexibility [25,26]. Lee et al. reported that introduction of a sulfur atom to an artemisinin moiety affords new derivatives which selectively control tumor related angiogenesis [27,28].

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Although different classes of cytotoxic artemisinin derivatives have been prepared, identification of the key factors contributing to their cytotoxicities and rational design of new classes of cytotoxic artemisinin analogues remain difficult. In this connection, it is of importance to conduct systematic structure-activity relationship (SAR) studies to assist the design and synthesis of new cytotoxic artemisinin derivatives in a rational manner.

Chalcone is an open chain flavonoid α, β-unsaturated carbonyl group and is one of the important compound groups derived from nature identified in *Angelica Keiskei* and exhibit interesting pharmacological activities [29,30]. Chemically, they are open-chained molecules bearing two aromatic rings linked by a three-carbon enone [31]. Both natural and synthetic chalcones exhibit various activities like antimicrobial, antimalarial, anti-inflammatory, anticancer, antioxidant and many more [29]. These activities are largely being attributed due to the unsaturated ketone moiety. Chalcone and its derivatives have been reported to be cytotoxic for cancer cells including leukemia cells [32].

Some of the new dimeric trioxane also have selective and potent anticancer activity [33]. Increasingly widespread evidence indicates that human cancer cells, richer than normal cells in iron-transport transferrin receptors [34,35], selectively activate trioxanes to produce various cytotoxic intermediates; this process is similar to that in the triggering of trioxanes by heme iron in malaria-infected human erythrocytes [8]. The anticancer properties of trioxanes have been reviewed [36–40]. Here we disclose that some of the new dimeric trioxane and monomers powerfully inhibit the growth (submicromolar IC_{50} values) of various cancer cells *in vitro*.

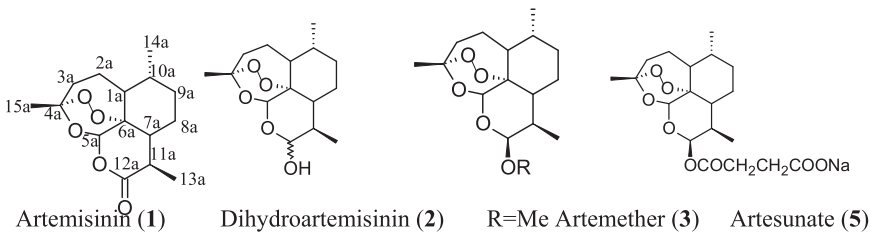
As more analogs were evaluated for antitumor activity, the unsymmetrical DHA acetal dimer was reported as being highly cytotoxic and more potent than cis-platin [1], while the symmetrical DHA acetal dimer also exhibited pronounced cytotoxic effects [41a]. In continuation of our work on artemsinin and chalcone in the area of antimalarial and anticancer drug discovery [41b-41e], these findings stimulated an interest in preparing additional DHA acetal dimers with various linkers. Synthesis and evaluations of many diversified dimers have been reported. However chalcone dimers are new and reported for the first time in this paper. We designed a new group of DHA monomers and dimers containing chalcone with a different substituent, linked by ether and chalcone as a linker in dimers and examined their *vitro* cytotoxic activities on human leukemia HL-60 cells, pancreatic cancer Mia PaCa-2 cells, prostate cancer PC-3 cells, colon cancer LS180 cells, hepatocellular carcinoma HEP G2. Since chalcones are known to have a wide variety of biological activities including anti-proliferative activity against leukemia cells, this study seems is an approach to develop drug like candidates.

In general, the cytotoxicities of artemisinin derivatives depends on type of linker [42,43] which decides efficient diffusion of compounds through membranes [44]. Yet, the effects of solubility, the stereochemistry (configuration and conformation) [42,45–51] and nature of functional groups of artemisinin derivatives could not be distinguished [47–51]. Therefore, structure modification might improve their anti-cancer activities. Several groups have performed modifications at the C-12 position of artemisinin and reported that the addition of an alkyl carbon chain (C_8H_{17} to $C_{16}H_{33}$) or a cyanoarylmethyl group significantly improved its antitumor activities [51]. In addition, it has been found that the endoperoxide in artemisinin is required for the cytotoxic activity. These data provide a rationale for the modification of artemisinin's structure in order to improve its antitumor activity.

2. Results and discussion

We have designed and synthesized a new group of DHA monomers and dimers containing chalcone moiety with a different substituent, linked by C-12 ether linkage/s. The synthetic pathways are shown in Scheme 1. Firstly, various chalcone based (basic structure: 1, 3-diphenyl-2-propene-1-one, Fig. 2.) analogues were synthesized by Claisen-Schmidt condensation based on a method reported previously [52,53]. In this process, an acetophenone was reacted with the corresponding aldehyde under acid/base catalyst. In the second step, the DHA prototype, 2-(12β-dihydroartemisinoxy)-ethyl bromide (**2P**) was synthesized according to a reported procedure [40], by the reaction of bromoethanol and DHA in presence of $BF_3.OEt_2$ in dichloromethane. The intermediate compound **2P** was crystallised with methanol and filtered as white crystals. Finally, in the third step the targeted artemisinin monomers **6**–**20** (using 1 eq. of **2P** and 1 eq. of respective chalcone) (Scheme 1.) and dimers, **21**–**25** (using 2 eq. of **2P** and 1 eq. of respective chalcone) (Scheme 2.) were successfully obtained by the reaction of the DHA prototype, **2P** and appropriate chalcones in the presence of K_2CO_3 and KI in *N*,*N*-dimethylformamide (DMF) at 60 °C, respectively. The products obtained were purified by silica gel column chromatography.

The stereochemistry (αH-12 or βH-12) of these compounds was confirmed by the application of ^{1}H NMR technique which analyzes the chemical shift of H-12 and the coupling constant between H-11 and H-12. The DHA aliphatic ethers are 12β-isomers as indicated by a chemical shift (4.60–4.90 ppm) and a small coupling constant (J = ~3.3 Hz) [28]. The geometrical configuration of the chalcone (α, β-unsaturated ketone) side chain was determined to be *trans* by observing the coupling constant (J = ~15.6 Hz) between H-α (H-2) and H-β (H-3). Among the synthesized compounds, **8**, **14**, **15**, **20** and **24** were found to be more active than parent DHA against tested human cancer cell lines. DHA derivatives were found to be most active in human leukemia cell lines with compounds **8**, **14**, **15**, **20** and **24** showed IC_{50} values less than 1 μM for 48 h whereas DHA has IC_{50} value of 2 μM at same time period. It is evident from Table 2 that a progressive increase in electron density on ring A/B of chalcone resulted in progressive enhancement in anticancer potency, with the para hydroxyl substituted hybrids **8** and **15**



Artemisinin (**1**) Dihydroartemisinin (**2**) R=Me Artemether (**3**) Artesunate (**5**)

R=Et Arteether (**4**)

Fig. 1. Artemisinin and its derivatives.

displaying the most promising activity (IC_{50} = 0.3 μM and IC_{50} = 0.4 μM respectively) followed by para hydroxyl, dimethoxy (**20**, IC_{50} = 0.5 μM). On the other hand, the replacement of ring A with heteroaromatic moieties like heterocyclic benzofuran ring led to only moderate activities (**14**, IC_{50} = 0.7 μM).

The presence of an electron withdrawing (Cl) group on ringA/B of chalcone resulted in loss of activity. In view of the immense pharmacological importance of hydroxy substituted chalcones, we were inspired to evaluate the anticancer profiles of various artemsinin-chalcone hybrids possessing a hydroxylated chalcone ring [29–32]. Gratifyingly, our above premise was confirmed, as the hydroxy artemsinin-chalcone hybrids were in general found to display comparatively enhanced potency with anticancer activity, progressively increasing upon an increase in electron density at chalcone ringA/B, (**8**, **15**, **20** and **14** with IC_{50} of 0.3, 0.4, 0.5, and 0.7 μM, respectively). In particular, **8** possessing free hydroxyl group in ring A, showed the best anticancer activity (IC_{50} = 0.3 μM) against the HL-60 cell line. Further the declining potency in dimethoxy positional hybrids **20** (IC_{50} = 0.5 μM) and **14** (IC_{50} = 0.7 μM) indicated the importance of dimethoxy substitution on chalcone ring for the most potent anticancer effect. The above observation was further reinforced when dimers **21**–**25** were synthesized by a similar procedure as given in Scheme 2, with **24** possessing electron donating group on chalcone ring, displayed higher potency (IC_{50} of 0.3 μM).

In order to discern the role of relative positions of rings A and B in the above scaffolds (A–C═CH–CO–B), the artemsinin-chalcone hybrids possessing chalcone moiety with reversal in rings A and B (A–CO–HC═C–B) were also evaluated for anticancer activity. Interestingly, such a study revealed the beneficial role of the presence of a strong electron withdrawing (carbonyl) group adjacent to the artemisinin moiety, as the artemsinin-chalcone hybrids possessing an exocyclic olefinic bond adjacent to the artemisinin moiety generally displayed lower activity, with **15** possessing an IC_{50} of 0.4 μM compared to **8** (IC_{50} = 0.3 μM). Having identified the core framework of an artemsinin-chalcone hybrid responsible for potent anticancer activity, we were encouraged to evaluate the effect of further diversification on the above motif by installing various functional groups on the chalcone rings. However, methyl group in the ring led to decreased activity, causing no change in potency (Table 1). Since progressive increments in the number of electron donating methoxy groups on chalcone rings led to molecules with progressively increasing anticancer potency, it is apparent that electron density in chalcone ring is a determinant of anticancer potency of artemsinin-chalcone. Hybrids with chalcones bearing free hydroxyl groups were most potent. The most potent compounds **8** with IC_{50} = 0.3 μM and **15** with IC_{50} = 0.4 μM, of the series, six and three times active than DHA (with IC_{50} = 2 μM) respectively were selected for further mechanistic work in human leukemia HL-60 cells.

Artesunic acid, the hemisuccinate derivative of dihydroartemisinin, is the only clinically useful water-soluble derivative of artemisinin. However, being a lactol ester, it is rapidly hydrolyzed back to dihydroartemisinin in aqueous alkaline solution.

The log P values for all of these dimers and monomers range between 5.34 and 7.45 and aqueous solubility with Log S values in the range of −4.76 to −6.58. The log P value of the active compounds **8** and **15** is 6.33. The log P of artemether (**2**) is 3.5. The derivatives are relatively polar. The compounds have aqueous solubility <10 mgL^{-1} at pH 7. Notably, compounds **8** and **15**, have solubility at pH 7 of >100 mgL^{-1}, with Log P value −5.96, a value which renders these compounds suitable for examination as an anticancer molecule, a purpose for which it would be more suited than DHA. Compound **8** and **15** possess anticancer activity similar to that of doxorubicin.

2.1. Dihydroarteminsin analogues inhibit cell proliferation in different cancer cell lines

Antiproliferative effect of dihydroarteminsin analogues **6**–**20** and dimers **21**–**25** were analyzed in different human cancer cell lines by using MTT assay. Most of the compounds inhibited cellular proliferation in different cancer cell lines and showed much potent activity than parent compound DHA (Table 1). Some highly potent DHA derivatives (**8**, **14**, **15**, **20** and **24**) were selected from series to further evaluate their IC_{50} values in human cancer cell lines HL-60, MiaPaCa-2, PC-3, HEP-G2 and LS180 cells for 48 h time period (Tables 2–6). These compounds showed most potent cytotoxicity in human leukemia HL-60 cells with IC_{50} values were less than 1 μM while the parent compound DHA showed IC_{50} value of around 2 μM.

2.2. Compounds *8* and *15* induced subG0 apoptotic population in human leukemia HL-60 cells

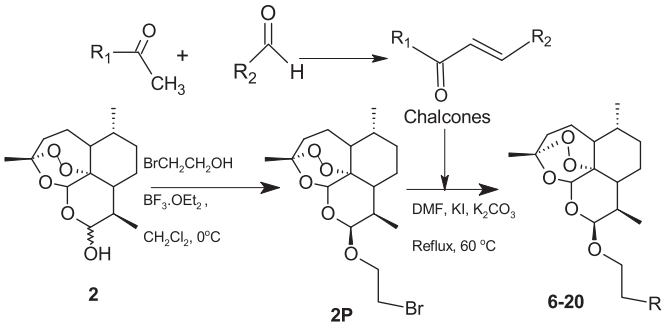
We have selected two most potent compounds **8** and **15** along with their parent compound DHA for further mechanistic work in human leukemia HL-60 cells. Compound **8** and **15** induced concentration dependent apoptotic DNA damage in cells treated for 24 h time period. Compound **8** treatment induced 28%, 95% and 96% apoptotic subG0 population at 0.5, 1 and 5 μM concentrations while **15** showed 9%, 65% and 70% DNA damage at same concentrations. DHA is less effective as compared to these analogues in inducing DNA damage as only 27% cells showed damaged DNA at 5 μM concentrations (Fig. 3).

2.3. Compounds *8* and *15* triggered mitochondrial potential (MMP) loss in human leukemia HL-60 cells

Perturbation in mitochondrial membrane causes the release of many factors that induced apoptotic signalling in cancer cells. We explored the effect of **8** and **15** along with comparative DHA treatment in inducing mitochondrial damage in HL-60 cells by using mitochondrial sensor dye rhodamine-123. Control cells that didn't get any compound treatment are almost healthy without any significant MMP loss whereas compound **8** treatment induced 27% MMP loss at 0.5 μM concentration, represented by M1 population in Fig. 4. At 1 and 5 μM the loss was 53% and 61% while **15** showed 50% and 58% MMP loss at 1 and 5 μM concentrations when given for 24 h time period. DHA is less effective then compounds **8** and **15** in triggering MMP loss as 1 and 5 μM concentrations of BA145 showed only 32 and 41% cells showed M1 population (Fig. 4).

2.4. Compounds *8* and *15* induced apoptotic bodies' formation in human leukemia HL-60 cells

To check the effect of compounds **8** and **15** on HL-60 cells nuclei, we performed hoechst staining in cells. Hoechst dye stains the condensed chromatin of apoptotic cells more brightly then chromatin of normal cells Compound **8** and **15** cause condensation of nuclei and induced morphological alterations in HL-60 cells in concentration dependent manner. Both compounds induced apoptotic bodies' induction even at 0.5 μM concentration when given for 24 h time period. DHA showed no any sign of apoptotic bodies' formation in HL-60 cells at these concentrations. Number of apoptotic bodies formed in HL-60 cells by compound **8** and **15** is much higher than DHA treatment (Fig. 5). Phase contrast microscopy of treated cells showed cell shrinkage and blebs formation in **8** and **15** treated HL-60 cells in dose dependent manner (Fig. 6).



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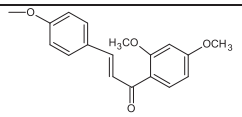
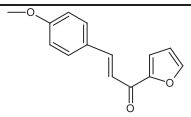
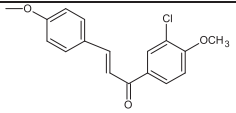
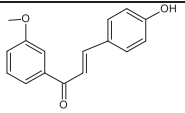
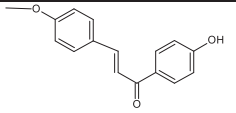
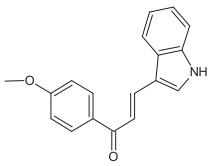
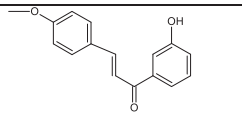
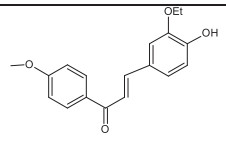
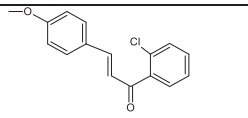
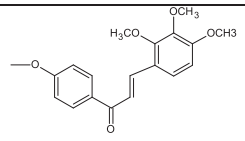
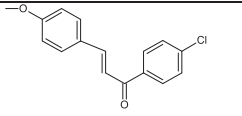
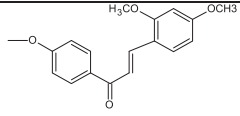
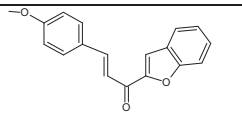
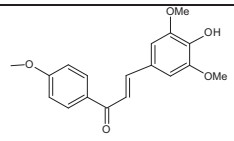
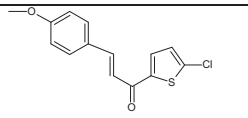
6		14	
7		15	
8		16	
9		17	
10		18	
11		19	
12		20	
13			

Scheme 1. Synthesis of chalcone based monomers of artemisinin.

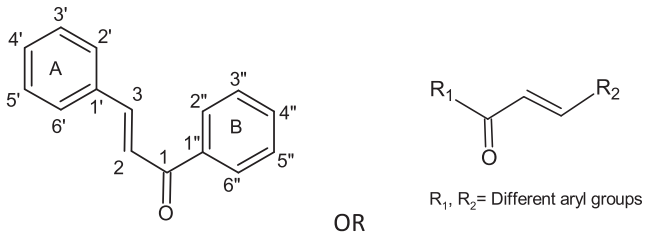
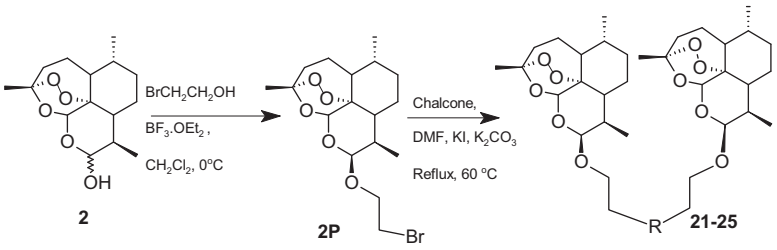
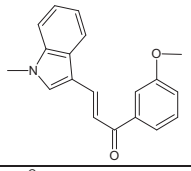
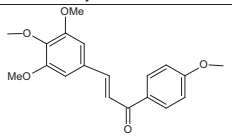
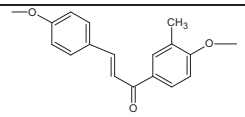
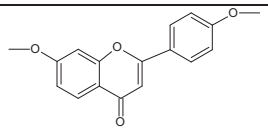
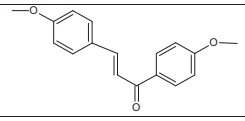


Fig. 2. Chalcone/chalcone based linkers.

Table 2
Potent cytotoxic artemisinin derivatives in leukemia HL-60 cells.

Compounds	0.5 μM	1 μM	10 μM	30 μM	IC_{50} value (μM)	SI (μM)
8	58.2	78.8	88.4	90.1	0.3	240
14	38	57.5	87.4	89.1	0.7	90
15	52.9	57.0	88.7	93.3	0.4	180
20	85.0	86.9	89.5	87.8	0.5	43
24	77.2	83.9	84.6	85.4	0.3	88
DHA (2)	36.0	48.1	63.0	91.0	2	63
Doxorubicin					0.3	255



Compounds	Structure, R=	Compounds	Structure, R=
21		**24**	
22		**25**	
23			

Scheme 2. Synthesis of chalcone based dimers of artemisinin.

Table 1
Cells growth inhibition by MTT assay (Time 48 h).

Comp.	HL-60 cells		Mia PaCa-2		PC-3		HEP G2		LS180	
	30 μM	50 μM	30 μM	50 μM	30 μM	50 μM	30 μM	50 μM	30 μM	50 μM
6	89.3	89.5	44.1	46.8	47.4	49.8	27.3	28.9	63.3	74.1
7	93.4	93.8	51.1	52.2	40.3	42.7	26.7	32.3	41.6	45.4
8	90.1	93.6	86.8	88.8	88.6	88.9	68.3	82.3	55.2	75.2
9	77.7	76.5	44.1	51.1	47.6	49.5	33.6	38.3	31.1	39.3
10	91.8	92.9	55.1	61.1	52.4	56.1	23.8	25.2	56.7	57.5
11	89.4	93.2	54.4	62.8	38.7	41.8	19.7	25.1	42.1	45.6
12	84.6	88.7	52.4	62.3	42.5	48.5	29.9	32.0	37.6	43.6
13	91.9	92.1	53.2	59.7	37.4	39.9	21.1	25.2	39.5	41.6
14	89.1	91.7	67.3	77.5	55.1	69.4	25.2	42.6	26.9	28.3
15	93.3	94.8	77.4	88.6	83.5	87.9	37.8	64.5	25.2	35.2
16	89.1	91.8	49.1	49.4	38.6	39.6	21.2	25.9	41.9	44.1
17	80.8	84.12	49.4	49.8	55.1	58.9	25.5	28.2	27.4	36.3
18	78.7	82.9	36.2	43.1	53.3	54.2	28.1	30.1	50.8	58.5
19	83.5	85.7	24.8	41.7	38.2	46.9	18.2	25.1	27.5	29.6
20	87.8	89.9	57.3	76.1	78.5	85.2	47.4	57.4	81.1	84.2
21	81.5	83.2	47.1	49.4	46.3	48.7	25.4	28.7	36.2	37.8
22	68.9	70.4	44.3	49.2	6.4	23.5	13.6	20.7	7.1	23.5
23	85.6	87.9	45.1	49.1	44.2	47.6	40.9	47.8	45.8	49.5
24	85.4	86.1	37.4	48.1	57.6	57.9	32.9	33.8	58.1	70.1
25	71.8	73.8	32.7	41.8	40.6	41.3	30.1	37.7	30.5	39.7
Artem	14.4	19.8	13	18	16	17.6	10.5	14.4	13.4	18.5
DHA	91	93	58	71	41	43	36	39	32	42

Table 3
Cytotoxic artemisinin derivatives in pancreatic cancer, Mia Pa Ca-2 cells.

Compounds	0.5 μM	1 μM	10 μM	30 μM	IC_{50} value (μM)	SI (μM)
8	6.2	9.1	16.8	68.3	22	88
14	18.4	19.1	26.2	39.8	>50	59
15	12.7	13.0	15.1	37.8	39	32
20	23.3	24.1	30	47.4	55.5	87
24	37.3	38.1	38.7	40.1	>50	100
DHA					58	12
Doxorubicin					1.75	>100

Table 4
Cytotoxic artemisinin derivatives in prostate cancer PC-3 cells.

Compounds	0.5 μM	1 μM	10 μM	30 μM	IC_{50} value (μM)	SI (μM)
8	19.3	25.4	41.5	88.6	14	82
14	15.1	15.8	43.4	58.1	24	94
15	15.2	22.8	29.6	83.5	18	80
20	32.4	34.5	51.2	78.5	15.15	56
24	34.5	45.9	50.2	57.6	10	210
DHA					>100	25
Doxorubicin					0.60	>73

Table 5
Cytotoxic artemisinin derivatives in hepatocellular carcinoma HEP G2 cells.

Compounds	0.5 μM	1 μM	10 μM	30 μM	IC_{50} value (μM)	SI (μM)
8	20.2	23.8	60.4	86.8	7	450
14	1.0	14.7	24.8	67.3	22	68
15	25.2	29.6	37.7	77.4	15	55
20	30.5	29.8	64.4	65.8	10.10	192
24	38.8	41.5	59.1	87.4	4	210
DHA					29	1.81
Doxorubicin					0.54	>100

Table 6
Cytotoxic artemisinin derivatives in colorectal carcinoma cells (LS180).

Compounds	0.5 μM	1 μM	10 μM	30 μM	IC_{50} value (μM)	SI (μM)
8	32.2	36.5	36.6	55.2	24	96
14	20	25.7	26.9	26.9	>50	>200
15	21.7	24.7	27.2	33.2	>50	>200
20	27	38.9	42.0	81.1	23.56	480
24	15.3	27.6	44.6	58.1	16	162
DHA					32	19
Doxorubicin					0.84	>100

3. Conclusion

We have prepared a new series of chalcone linker based monomers and dimers of artemisinin, several of which have shown better anticancer activity than dihydroatemisinin against HL-60 cell line. Monomers **8** with $IC_{50} = 0.3$ μM and **15** with $IC_{50} = 0.4$ μM, the most active compounds of the series, are six and three times active than DHA (with $IC_{50} = 2$ μM) respectively. The resulting artemisinin derivatives showed potent activity against cancer cell lines with high selectivity Index.

4. Materials and methods

4.1. General methods

Melting point was determined on a Toshniwal melting point apparatus and is uncorrected. IR spectra were recorded on a Perkin Elmer 1719 FT-IR spectrophotometer. NMR spectra were obtained in acetone-d_6 on a Bruker Avance, 300 and 400 MHz instrument using TMS as internal standard. The chemical shift values are reported in ppm and coupling constants in Hz. ESI-MS spectra were recorded on a Perkin Elmer Turbo Mass/Shimadzu LC-MS. TLC analyses were carried out on precoated silica gel 60 F_{254} plates (Merck) using solvent system, hexane: ethyl acetate (6:4). The compounds were visualized by either exposure of TLC plates to I_2 vapours or by spraying with vanillin-sulfuric acid reagent, followed by heating at 110 °C for 15 min. Si-gel, 60–120 mesh (spectrochem) was used in the column chromatography for the purification of metabolites. The compounds were identified by their spectral IR, ID (^{1}H, ^{13}C, DEPT) and 2D (COSY, HSQC, HMBC) NMR and ESIMS analysis. The purity of all synthesized compounds was determined by HPLC method. HPLC analysis was carried out on a reversed-phase waters 30 mm × 4.6 mm, 5 μm C18 column maintained at ambient temperature with a flow rate of 0.4 mL/min and 10 μL samples were injected in all experiments. The retention time was expressed in min at the UV detection of 240 nm. The mobile phase was composed of MeCN/H_2O (7:3, v/v). According to HPLC analysis, the purity of all compounds is >95%.

4.2. General procedure for the synthesis of artemisinin monomers/dimers (6–25)

4.2.1. Synthesis of 2-(12β-dihydroartemisinoxy) ethyl bromide (DHA-prototype, 2P)

$BF_3 \cdot Et_2O$ (16 mL) was added to a solution of DHA (3, 20.0 g, 70.4 mmol) and 2-bromoethanol (13.1 g, 105.6 mmol) in 100 ml of CH_2Cl_2 at 0 °C. The mixture was stirred at room temperature until the reaction completed. The reaction mixture was washed with saturated $NaHCO_3$ solution (100 ml), water (100 ml) and brine (100 ml). The organic layer was dried and concentrated. The residue was recrystallized to afford white crystals of compound **2P** (26.17 g, 90.0%) as, mp 154–157 °C; ^{1}H NMR (300 MHz, Acetone-d_6): δ 0.87 (3H, d, $J = 7.5$ Hz, H_3-13), 0.90 (3H, d, $J = 6.5$ Hz, H_3-14), 1.31 (3H, s, H_3-15), 2.41 (1H, m, H-11a), 4.77 (1H, d, $J = 3.4$ Hz, Ha-1a′), 3.94 (1H, td, $J = 9.3$, 4.1 Hz, 1b′), 3.68 (2H, m, H_2-2a′), 4.83 (1H, d, $J = 3.5$ Hz); 5.43 (1H, s, H-5); ESI-MS: m/z 413 $[M+Na]^+$, molecular formula $C_{17}H_{27}O_5Br$.

4.2.2. Synthesis of linker moieties (chalcones)

Chalcone linkers for synthesized artemisinin monomers and dimers were synthesized as per method reported in literature. A mixture of the acetophenone (10 mmol) and the appropriate aldehyde (11 mmol) in anhydrous MeOH (30 mL) was stirred at room temperature for 5 min. Then, $SOCl_2$ (2 ml) was added. The reaction mixture was stirred at room temperature for 2 h. Added water (30 mL). The precipitate obtained was filtered and recrystallized from 95% EtOH to yield crystals of chalcones.

4.2.3. Synthesis of DHA monomers and dimers

K_2CO_3 (0.03 mol) was added to a stirred solution of 2-(12β-dihydroartemisinoxy)-ethyl bromide (**2P**, 10 mmol for monomers and 20 mmol for dimers) in DMF (50 mL) at 60 °C for 30 min followed by adding appropriate chalcones: 4′-hydroxy-2, 4-dimethoxychalcone (for **6**); 3-chloro-4′-hydroxy-4-methoxychalcone (for **7**); 4, 4′-dihydroxychalcone (for **8**, **23**); 3, 4′-dihydroxychalcone (for **9, 15**); 2-chloro-4′-hydroxychalcone (for **10**); 4-chloro-4′-hydroxychalcone (for **11**); 1-(1″-benzofuranyl)-3-(4′-hydroxyphenyl)-2-propen-1-one (for **12**); 1-(3″-chloro-1″-thienyl)-3-(4′-hydroxyphenyl)-2-propen-1-one (for **13**); 1-(1″-furanyl)-3-(4′-hydroxyphenyl)-2-propen-1-one (for **14**); 1-(4″-hydroxyphenyl)-3-(1′-indolyl)-2-propen-1-one (for **16**); 3′-ethoxy-4, 4′-dihydroxychalcone (for **17**); 4-hydroxy-2′, 3′, 4’-trimethoxychalcone (for **18**); 4-hydroxy-2′, 4′ -dimethoxychalcone (for **19**); 4,

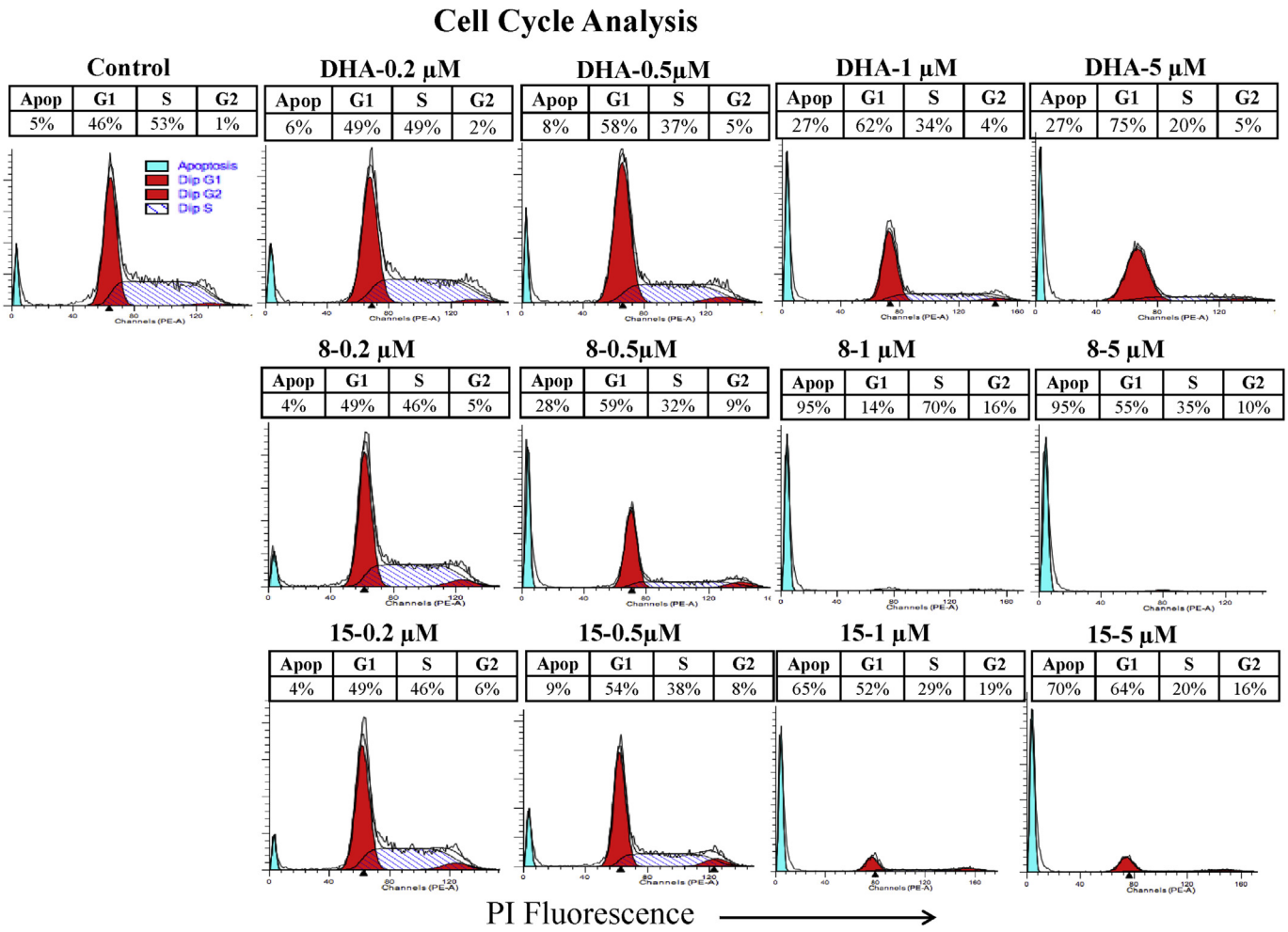


Fig. 3. Compounds DHA, **8** and **15** induced apoptotic DNA damage in human leukemia HL-60 cells. HL-60 cells (0.5×10^6 cells/ml/12well plate) were treated with 0.2, 0.5, 1 and 5 μM concentration of compounds DHA, **8** and **15** for 24 h time period. Cells were washed with PBS twice, fixed with 70% ethanol and stained with propidium iodide (PI) to determine DNA fluorescence by flow cytometer as described in material and methods. The fraction of cells for hypo-diploid (sub-G0, <2n DNA) population analyzed from FL2-A vs. cell counts by using ModFit software is shown. Data is representative of one of three similar experiments.

4′-dihydroxy, 3′, 5′-dimethoxychalcone (for **20, 24**); 1-(3″-hydroxyphenyl)-3-(1′-indolyl)-2-propen-1-one (for **21**); 4, 4′-dihydroxy-3-methylchalcone (for **22**); 7, 4′-dihydroxyflavone (LTG, for **25**) (9 mmol) and KI as catalysts and stirring further the reaction at 120 °C for 5–6 h. After completion of the reaction, the reaction mixture was washed with 5% NaOH (30 ml) and water (30 ml) and extracted with ethyl acetate. The organic layer (ethyl acetate) was dried (Na_2SO_4) and concentrated *in vacuo* to furnish the crude products. Pure DHA monomers and dimers (**6**–**25**) were obtained by column chromatography (silica gel) using hexane-ethyl acetate (8-5:2-5 v/v) as eluent.

*4.2.3.1. 3-[4′-{1a′-(12aβ-dihydroxyartemisinoxy)}-ethoxyphenyl]-1-(2″, 4″-dimethoxyphenyl)-2-propen-1-one (**6**).* Elution of the column with *n*-hexane-ethyl acetate (70:20) yielded a viscous compound, 90% (w/w) yield; IR ν ^{max}(neat): 1654 (chalcone), 1253, 1169, 1109 (ether), 1601, 1459, 1362, 1026, 984 (aromatics) cm^{-1}; ^{1}H, COSY- NMR (300 MHz, Acetone-d_6): δ 0.84 (3H, d, *J* = 9.3 Hz, H_3-13a), 0.88 (3H, d, *J* = 7.5 Hz, H_3-14a), 1.21 (3H, s, H_3-15a), 2.46 (1H, m, H-11a), 3.88, 3.95 (3H each, s, 2×OCH_3), 3.79 (1H, m, Ha-1a′), 4.05 (1H, m, Hb-1a′), 4.27 (2H, d, *J* = 6.3 Hz, H_2-2a′), 4.78 (1H, d, *J* = 3.0 Hz, αH-12a), 5.42 (1H, s, H-5a), 6.60 (1H, d, *J* = 2.4 Hz, H-3″), 6.64 (1H, dd, *J* = 8.7, 2.1 Hz, H-5″), 7.02 (2H, dd, *J* = 8.7, 1.8 Hz, H-3′, H-5′), 7.48 (1H, d, *J* = 15.9 Hz, H-2), 7.58 (1H, d, *J* = 15.9 Hz, H-3), 7.64 (1H, m, H-6″), 7.66 (2H, d, *J* = 8.7 Hz, H-2′, H-6′); ^{13}C, DEPT-NMR (75 MHz, Acetone-d_6): δ 12.82 (C-13a), 20.18 (C-14a), 24.64 (C-8a), 25.07 (C-2a), 25.75 (C-15a), 31.29 (C-11a), 34.96 (C-9a), 36.68 (C-3a), 37.57 (C-10a), 44.90 (C-7a), 53.00 (C-1a), 55.51, 55.79 (2×OCH_3), 66.54 (C-1a′), 67.92 (C-2a′), 81.01 (C-6a, q), 87.91 (C-5a), 98.79 (C-3″), 101.99 (C-12a), 103.88 (C-4a, q), 106.13 (C-5″), 115.54 (C-5′, C-5′), 122.73 (C-1″, q), 125.58 (C-2), 128.60 (C-1′, q), 130.34 (C-2′, C-6′), 132.68 (C-6″), 141.33 (C-3), 160.89 (C-2″, q), 161.17 (C-4′, q), 164.64 (C-4″, q), 189.52 (C-1, q); HR-MS (*m*/*z*) $[M+H]^+$ (ESI^+) calcd for $C_{34}H_{43}O_9$, 595.2901; found, 595.2903. ESI-MS(positive): *m*/*z* 595 $[M+H]^+$, 617 $[M+Na]^+$ and (negative): 593 $[M-H]^+$, molecular formula $C_{34}H_{42}O_9$.

*4.2.3.2. 1-(3″-Chloro-4″-methoxyphenyl)-3-[4′-{1a′-(12aβ-dihydroxyartemisinoxy)}-ethoxyphenyl]-2-propen-1-one (**7**).* Elution of the column with *n*-hexane-ethyl acetate (70:20) yielded a colourless solid, 90% (w/w) yield; mp. 62 °C; IR ν ^{max}(neat): 1655 (chalcone), 1254, 1173, 1107 (ether), 1595, 1509, 1469, 1203, 1029, 984, (aromatics), 788 (Cl group) cm^{-1}; ^{1}H, COSY- NMR (300 MHz, Acetone-d_6): δ 0.87 (6H, d, *J* = 6.9 Hz, H_3-13a, H_3-14a), 1.30 (3H, s, H_3-15a), 2.48 (1H, m, H-11a), 3.99 (3H, s, OCH_3), 4.03 (2H, m, H_2-1a′), 4.26 (2H, t, *J* = 4.2 Hz, H_2-2a′), 4.78 (1H, d, *J* = 3.3 Hz, αH-12a), 5.42 (1H,

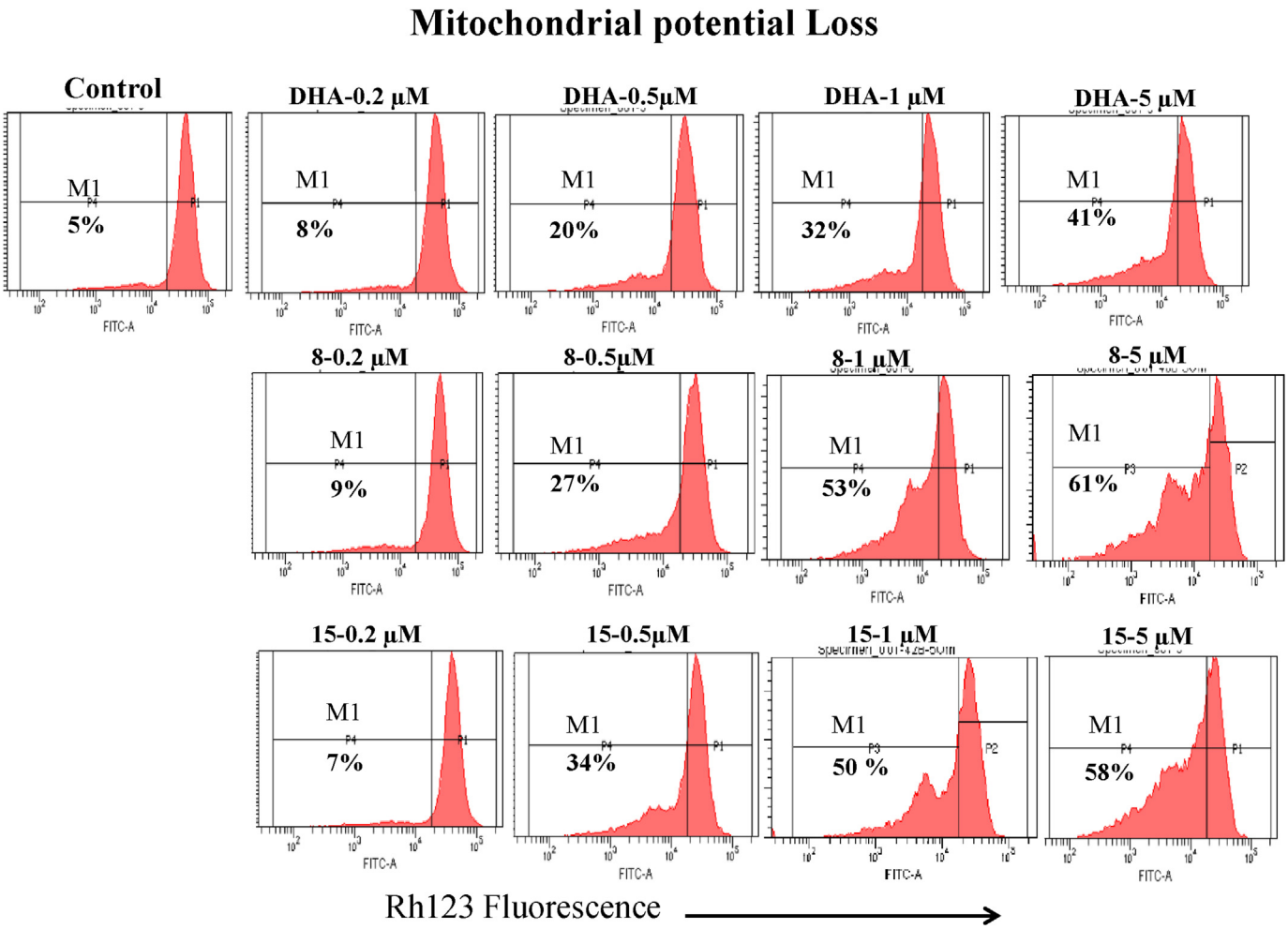


Fig. 4. Compounds DHA, **8** and **15** trigger mitochondrial potential loss in HL-60 cells. Cells (0.5×10^6 cells/ml/12well plate) were treated 0.2, 0.5, 1 and 5 μM concentration of compounds DHA, **8** and **15** for 24 h time period. Rhoda mine-123dye was added 40 min prior to experiment termination, cells were collected, washed with PBS twice and analyzed flow cytometrically on FL-1 channel. Decrease in Rh-123 fluorescence is represented by M1 population in figure. Data is representative of one of three similar experiments.

s, H-5a), 7.01 (2H, dd, J = 8.7, 1.8 Hz, H-3′, H-5′), 7.22 (1H, d, J = 8.7 Hz, H-5″), 7.71 (1H, d, J = 16.5 Hz, H-2), 7.74 (1H, s, H-2″), 7.77 (2H, d, J = 8.7 Hz, H-2′, H-6′), 8.11 (1H, d, J = 8.7 Hz, H-6″), 8.13 (1H, d, J = 16.5 Hz, H-3); ^{13}C, DEPT- NMR (75 MHz, Acetone-d_6): δ12.81 (C-13a), 20.18 (C-14a), 24.64 (C-8a), 25.07 (C-2a), 25.74 (C-15a), 31.29 (C-11a), 34.95 (C-9a), 36.66 (C-3a), 37.58 (C-10a), 44.89 (C-7a), 52.99 (C-1a), 56.46 (OCH_3), 66.50 (C-1a′), 67.96 (C-2a′), 81.01 (C-6a, q), 87.91 (C-5a), 101.98 (C-12a), 103.88 (C-4a, q), 112.38 (C-5″), 115.43, 116.29 (C-3′/C-5′), 119.36 (C-2), 122.71 (C-3″, q), 128.27 (C-1′, q), 129.55 (C-6″), 130.63 (C-2″), 130.94, 131.14 (C-2′/C-6′), 131.14 (C-2″), 132.30 (C-1″, q), 144.24 (C-3), 158.97 (C-4″, q), 161.53 (C-4′, q), 186.76 (C-1, q); HR-MS (m/z) $[M+H]^+$ (ESI^+) calcd for $C_{33}H_{40}O_8Cl$, 599.2406; found, 599.2407. ESI-MS(positive): m/z 599 $[M+H]^+$, 621 $[M+Na]^+$, 637 $[M+K]^+$, molecular formula $C_{33}H_{39}O_8Cl$.

*4.2.3.3. 3-[4′-{1a′-(12aβ-dihydroxyartemisinoxy)}-ethoxyphenyl]-1-(4″-hydroxyphenyl)-2-propen-1-one (**8**).* Elution of the column with *n*-hexane-ethyl acetate (70:30) yielded a viscous compound, 55% (w/w) yield; IR ν ^{max}(neat): 3567 (OH), 1654 (chalcone), 1256, 1173, 1107 (ether), 1602, 1514, 1464, 1201, 1031, 981, (aromatics) cm^{-1}; ^{1}H, COSY- NMR (300 MHz, Acetone-d_6): δ 0.81 (6H, d, J = 7.20 Hz, H_3-13a, H_3-14a), 1.17 (3H, s, H_3-15a), 2.42 (1H, m, H-11a), 3.70 (1H, m, H_a-1a′), 4.09 (1H, m, H_b-1a′), 4.19 (2H, d, J = 4.2 Hz, H_2-2a′), 4.71 (1H, d, J = 3.0 Hz, αH-12a), 5.36 (1H, s, H-5a), 6.84 (2H, d, J = 8.4 Hz, H-3′, H-5′), 7.00 (2H, d, J = 8.7 Hz, H-3″, H-5″), 7.59 (1H, d, J = 15.3 Hz, H-2), 7.62 (1H, d, J = 15.3 Hz, H-3), 7.68 (2H, d, J = 8.7 Hz, H-2′, H-6′), 8.05 (2H, d, J = 8.7 Hz, H-2″, H-6″); ^{13}C, DEPT-NMR (75 MHz, Acetone-d_6): δ 12.60 (C-13a), 20.13 (C-14a), 24.64 (C-8a), 25.60 (C-2a), 25.72 (C-15a), 31.30 (C-11a), 34.96 (C-9a), 36.76 (C-3a), 37.58 (C-10a), 44.91 (C-7a), 53.02 (C-1a), 66.50 (C-1a′), 67.94 (C-2a′), 81.02 (C-6a, q), 87.93 (C-5a), 102.00 (C-12a), 103.89 (C-4a, q), 114.82 (C-3′, C-5′), 116.29 (C-3″, C-5″), 119.07 (C-2), 127.19 (C-1′, q), 130.93, 131.02 (C-2'/C-6′, C-2"/C-6″), 131.89 (C-1″, q), 144.14 (C-3), 160.36 (C-4″, q), 163.45 (C-4′, q), 187.78 (C-1, q); HR-MS (m/z) $[M+H]^+$ (ESI^+) calcd for $C_{32}H_{39}O_8$, 551.2639; found, 551.2640. ESI-MS(positive): m/z 551 $[M+H]^+$, 573 $[M+Na]^+$, 589 $[M+K]^+$, molecular formula $C_{32}H_{38}O_8$.

*4.2.3.4. 3-[4′-{1a′-(12aβ-dihydroxyartemisinoxy)}-ethoxyphenyl]-1-(3″-hydroxyphenyl)-2-propen-1-one (**9**).* Elution of the column with *n*-hexane-ethyl acetate (70:40) yielded a viscous compound,, 38% (w/w) yield, more polar than monomer **60**; IR ν ^{max}(neat): 3425 (OH), 1661 (chalcone), 1252, 1175, 1108 (ether), 1590, 1510, 1443, 1030, 985, (aromatics) cm^{-1}; ^{1}H, COSY- NMR (300 MHz, Acetone d_6): δ 0.79 (6H, d, J = 6.0 Hz, H_3-13a, H_3-14a), 1.12 (3H, s, H_3-15a), 2.41 (1H, m, H-11a), 3.69 (1H, m, H_a-1a′), 4.04 (1H, m, H_b-1a′), 4.22 (2H, d, J = 4.2 Hz, H_2-2a′), 4.72 (1H, d, J = 3.0 Hz, αH-12a), 5.35 (1H,

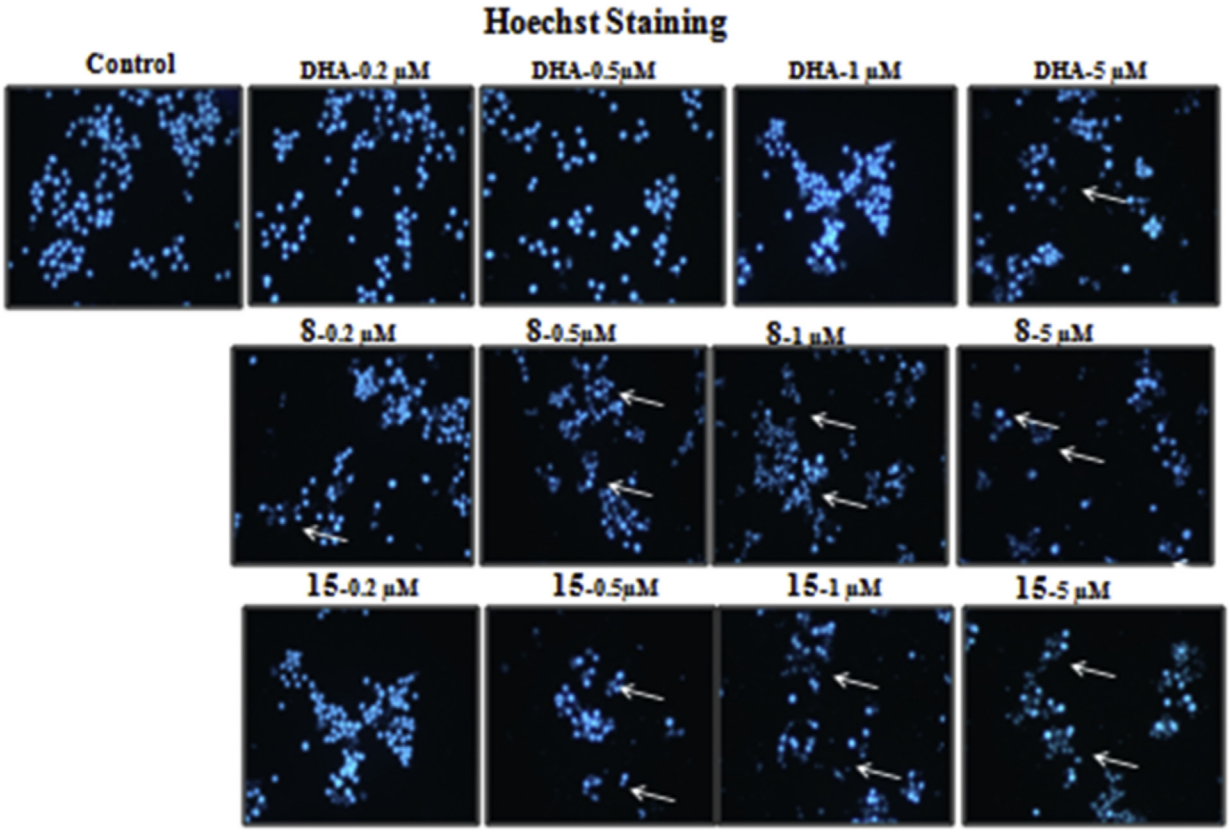


Fig. 5. Compounds DHA, **8** and **15** induced apoptotic bodies formation in human leukemia HL-60 cells. Cells (0.5×10^6/2ml/12 well plate) were treated with 0.2, 0.5, 1 and 5 μM concentration of compounds for 24 h time period. Cells were collected, fixed and then after stained with Hoechst 33258 dye as described in material and methods. Cells were observed under fluorescence microscopy at 20×. Compounds **8** and **15** triggered more apoptotic bodies formation as compared to DHA treatment in HL-60 cells as indicated by white arrows in the figure. Data are one of three similar experiments.

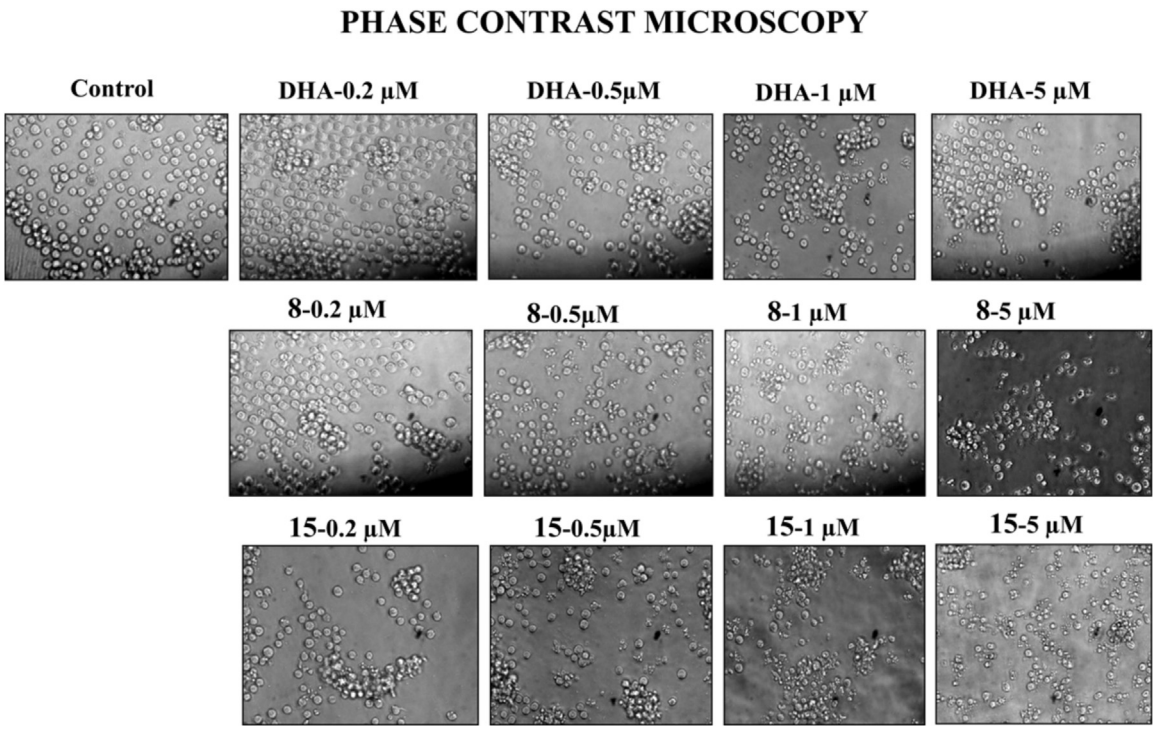


Fig. 6. Effect of compounds DHA, **8** and **15** on the morphology of HL-60 cells. Cells (0.5×10^6/2ml/12 well plate) were treated with indicated concentrations of compounds DHA, **8** and **15** for 24 h time period. After treatment, cells were photographed under phase contrast microscope at 20×. Data are one of three similar experiments.

s, H-5a), 6.97 (2H, d, $J = 8.4$ Hz, H-3′, H-5′), 7.12 (1H, m, H-4″), 7.39 (2H, m, H-2″, H-5″), 7.60 (1H, d, $J = 15.9$ Hz, H-2), 7.66 (4H, m, H-3, H-2′, H-6′, H-6″), 7.83 (1H, brs, 3′-OH); ^{13}C, DEPT- NMR (75 MHz, Acetone-d_6): δ 12.78 (C-13a), 20.14 (C-14a), 24.64 (C-8a), 25.05 (C-2a), 25.72 (C-15a), 31.30 (C-11a), 34.97 (C-9a), 36.68 (C-3a), 37.58 (C-10a), 44.93 (C-7a), 53.04 (C-1a), 66.52 (C-1a′), 67.98 (C-2a′), 81.00 (C-6a, q), 87.91 (C-5a), 102.00 (C-12a), 103.85 (C-4a, q), 114.52 (C-2"), 115.47 (C-3′, C-5′), 119.48, 120.09, 121.24 (C-2,/C-4″,/C-6″), 128.29 (C-1′, q), 130.10 (C-5″), 130.89 (C-2′, C-6′), 140.37 (C-1″, q), 144.32 (C-3), 159.82 (C-3″, q), 161.63 (C-4′, q), 189.04 (C-1, q); HR-MS (*m*/*z*) [M + H]+ (ESI+) calcd for $C_{32}H_{39}O_8$, 551.2639; found, 551.2639. ESI-MS(positive): *m*/*z* 551 $[M+H]^+$, (Negative): 549 $[M-H]^-$, molecular formula $C_{32}H_{38}O_8$.

*4.2.3.5. 1-(2″-Chlorophenyl)-3-[4′-{1a′-(12aβ-dihydroxyartemisinoxy)}-ethoxyphenyl]-2-propen-1-one (**10**).* Elution of the column with *n*-hexane-ethyl acetate (70:30) yielded a viscous compound,, 75% (w/w) yield,; IR ν ^{max}(neat): 1656 (chalcone), 1250, 1173, 1102 (ether), 1591, 1519, 1440, 1029, 982, (aromatics), 783 (Cl group) cm^{-1}; ^{1}H, COSY- NMR (300 MHz, Acetone d_6): δ 0.81 (6H, d, $J = 7.5$ Hz, H_3-13a, H_3-14a), 1.28 (3H, s, H_3-15a), 2.49 (1H, m, H-11a), 3.96 (1H, m, H_a-1a′), 4.01 (1H, m, H_b-1a′), 4.21 (2H, m, H_2-2a′), 4.71 (1H, d, $J = 3.0$ Hz, αH-12a), 5.32 (1H, s, H-5a), 6.89 (2H, d, $J = 7.2$ Hz, H-3′, H-5′), 7.01 (2H, m, H-4″, H-5″), 7.33 (1H, d, $J = 15.6$ Hz, H-2), 7.46 (4H, m Hz, H-2′, H-6′, H-3, H-6″); ^{13}C, DEPT- NMR (75 MHz, Acetone-d_6): δ 17.77 (C-13a 20.14 (C-14a), 24.63 (C-8a), 25.05 (C-2a), 25.72 (C-15a), 31.29 (C-11a), 34.96 (C-9a), 36.67 (C-3a), 37.58 (C-10a), 44.90 (C-7a), 53.03 (C-1a), 66.50 (C-1a′), 67.99 (C-2a′), 80.99 (C-6a, q), 87.91 (C-5a), 102.00 (C-12a), 103.86 (C-4a, q), 115.58 (C-3′, C-5′), 124.55 (C-2), 127.50 (C-5″), 129.57 (C-3″), 127.67 (C-1′), 131.13 (C-2′, C-6′), 131.56 (C-6″), 131.66 (C-4″), 139.00 (C-2″, q), 140.09 (C-1″, q), 146.30 (C-3), 161.93 (C-4′, q), 193.15 (C-1, q); HR-MS (*m*/*z*) $[M+H]^+$ (ESI^+) calcd for $C_{32}H_{38}O_7Cl$, 569.2300; found, 569.2300. ESI-MS(positive): *m*/*z* 569 $[M+H]^+$, 591 $[M+Na]^+$, molecular formula $C_{32}H_{37}O_7Cl$.

*4.2.3.6. 1-(4″-chlorophenyl)-3-[4′-{1a′-(12aβ-dihydroxyartemisinoxy)}-ethoxyphenyl]-2-propen-1-one (**11**).* Elution of the column with *n*-hexane– ethyl acetate (70:30) yielded a viscous compound, 75% (w/w); IR ν ^{max} (neat): 1647 (chalcone), 1250, 1170, 1107 (ether), 1595, 1511, 1435, 1027, 983, (aromatics), 766 (Cl group) cm^{-1}; ^{1}H, COSY- NMR (300 MHz, Acetone d_6): δ 0.75 (6H, d, $J = 6.9$ Hz, H_3-13a, H_3-14a), 1.21 (3H, s, H_3-15a), 2.39 (1H, m, H-11a), 3.62 (1H, m, H_a-1a′), 3.96 (1H, m, H_b-1a′), 4.14 (2H, t, 4.2 Hz, H_2-2a′), 4.66 (1H, d, $J = 2.7$ Hz, αH-12a), 5.30 (1H, s, H-5a), 6.89 (2H, d, $J = 8.4$ Hz, H-3′, H-5′), 7.42 (2H, d, $J = 8.4$, H-2″, H-6″), 7.53 (3H, m, H-2′, H-6′, H-2), 7.66 (4H, d, $J = 15.00$ Hz, 3), 8.00 (2H, d, $J = 8.7$ Hz, H-3″, H-5″); ^{13}C, DEPT- NMR (75 MHz, Acetone-d_6): δ 12.82 (C-13a), 20.19 (C-14a), 24.65 (C-8a), 25.07 (C-2a), 25.77 (C-15a), 31.29 (C-11a), 34.97 (C-9a), 36.68 (C-3a), 37.60 (C-10a), 44.90 (C-7a), 52.99 (C-1a), 66.53 (C-1a′), 67.99 (C-2a′), 81.03 (C-6a, q), 87.94 (C-5a), 102.02 (C-12a), 103.92 (C-4a. q), 115.49 (C-3′, C-5′), 119.59 (C-2), 128.14 (C-1′,q), 129.16 (C-3″, C-5″), 130.50 (C-2′, C-6′), 131.10 (C-2″, C-6″), 137.59 (C-1″, q), 138.60 (C-4″, q), 144.91 (C-3), 161.74 (C-4′, q), 188.32 (C-1, q); HR-MS (*m*/*z*) $[M+H]^+$ (ESI^+) calcd for $C_{32}H_{38}O_7Cl$, 569.2300; found, 569.2300. ESI-MS(positive): *m*/*z* 569 $[M + H]^+$, molecular formula $C_{32}H_{37}O_7Cl$.

*4.2.3.7. 1-(1″-benzofuranyl)-3-[4′-{1a′-(12aβ-dihydroxyartemisinoxy)}-ethoxyphenyl]-2-propen-1-one (**12**).* Elution of the column with *n*-hexane– ethyl acetate (70:20) yielded a colourless solid, 78% (w/w); mp. 65 °C; IR ν ^{max} (neat): 1659 (chalcone), 1254, 1165 1108 (ether), 1592, 1510, 1452, 1031, 984, (aromatics), 750 (furan moiety) cm^{-1}; ^{1}H, COSY- NMR (300 MHz, Acetone d_6): δ 0.81 (3H, d, $J = 7.5$ Hz, H_3-13a), 0.82 (3H, d, $J = 6.0$ Hz, H_3-14a), 1.28 (3H, s, H_3-15a), 2.43 (1H, m, H-11a), 3.73 (1H, m, H_a-1a′), 3.96 (1H, m, H_b-1a′), 4.22 (2H, t, $J = 3.9$ Hz, H_2-2a′), 4.72 (1H, d, $J = 3.6$ Hz, αH-12a), 5.36 (1H, s, H-5a), 6.98 (2H, dd, $J = 8.7$, 1.2 Hz, H-3′, H-5′), 7.29 (1H, dd, $J = 7.8$, 0.9 Hz, H-5″), 7.46 (1H, dd, $J = 8.1$, 1.2 Hz, H-6″), 7.49 (1H, d, $J = 8.1$ Hz, H-7″), 7.61 (1H, d, $J = 15.8$ Hz, H-2), 7.73 (3H, dd, $J = 8.7$, 2.1 Hz, H-2′, H-6′, H-4 ″), 7.70 (1H, d, $J = 15.6$ Hz, H-3), 7.87 (1H, brs, H-2″); ^{13}C, DEPT- NMR (75 MHz, Acetone-d_6): δ 12.98 (C-13a), 20.15 (C-14a), 24.64 (C-8a), 25.06 (C-2a), 25.73 (C-15a), 31.29 (C-11a), 34.96 (C-9a), 36.67 (C-3a), 37.59 (C-10a), 44.91 (C-7a), 53.02 (C-1a), 66.50 (C-1a′), 67.99 (C-2a′), 81.00 (C-6a, q), 87.92 (C-5a), 101.99 (C-12a), 103.87 (C-4a, q), 112.50 (C-2″), 113.46 (C-7″), 115.54 (C-3′, C-5′), 119.68 (C-4″), 123.79 (C-5″), 124.31 (C-6″), 127.92 (C-1′, q), 127.96 (C-3″, q), 128.50 (C-2), 131.04 (C-2′, C-6′), 143.92 (C-3), 154.50 (C-8″, q), 156.09 (C-1″, q), 161.86 (C-4′, q), 179.00 (C-1, q); HR-MS (*m*/*z*) $[M+H]^+$ (ESI^+) calcd for $C_{34}H_{39}O_8$, 575.2639; found, 575.2642. ESI-MS(positive): *m*/*z* 597 $[M+Na]^+$, (Negative): 573 $[M-H]^-$, molecular formula $C_{34}H_{38}O_8$.

*4.2.3.8. 1-(3″-Chloro-2″-thienyl)-3-[4′-{1a′-(12aβ-dihydroxyartemisinoxy)}-ethoxyphenyl]-2-propen-1-one (**13**).* Elution of the column with *n*-hexane-ethyl acetate (70:30) yielded a viscous compound, 85% (w/w); IR ν ^{max} (neat): 1647 (chalcone), 1258, 1174, 1107 (ether), 1588, 1511, 1028, 984, (aromatics), 1421, 826 (thiophene group), 789 (Cl group) cm^{-1}; ^{1}H, COSY- NMR (300 MHz, Acetone d_6): δ 0.78 (3H, d, $J = 9.3$ Hz, H_3-13a), 0.82 (3H, d, $J = 6.0$, H_3-14a), 1.28 (3H, s, H_3-15a), 2.44 (1H, m, H-11a), 3.65 (1H, m, H_a-1a′), 3.98 (1H, m, H_b-1a′), 4.20 (2H, d, $J = 4.5$ Hz, H_2-2a′), 4.72 (1H, d, $J = 3.0$ Hz, αH-12a), 5.35 (1H, s, H-5a), 6.96 (2H, d, $J = 8.7$ Hz, H-3′, H-5′), 7.12 (1H, d, $J = 3.6$, H-2″), 7.76 (3H, m, H-2, H-2′, H-6′), 7.53 (1H, d, $J = 15.9$ Hz, H-3), 7.94 (1H, d, $J = 4.2$ Hz, H-3″); ^{13}C, DEPT-NMR (75 MHz, Acetone-d_6): δ 12.76 (C-13a), 20.14 (C-14a), 24.63 (C-8a 25.06 (C-2a), 25.71 (C-15a), 31.29 (C-11a), 34.97 (C-9a), 36.66 (C-3a), 37.59 (C-10a), 44.91 (C-7a), 53.02 (C-1a), 66.49 (C-1a′), 67.98 (C-2a′), 80.98 (C-6a, q), 87.91 (C-5a), 101.98 (C-12a), 103.85 (C-4a, q), 115.48 (C-3′, C-5′), 118.50 (C-2), 127.93 (C-1′, q), 128.77 (C-5″), 131.04 (C-2′, C-6′), 132.29 (C-2″), 138.44 (C-3″, q), 145.79 (C-1″, q), 144.08 (C-3), 161.82 (C-4′, q), 180.94 (C-1, q); HR-MS (*m*/*z*) $[M + H]^+$ (ESI^+) calcd for $C_{30}H_{36}O_7SCl$, 575.1864; found, 575.1864. ESI-MS(positive): *m*/*z* 575 $[M+H]^+$, 597 $[M+Na]^+$, 613 $[M+K]^+$, molecular formula $C_{30}H_{35}O_7SCl$.

*4.2.3.9. 3-[4′-{1a′-(12aβ-dihydroxyartemisinoxy)}-ethoxyphenyl]-1-(1″-furanyl)-2-propen-1-one (**14**).* Elution of the column with *n*-hexane-ethyl acetate (70:25) yielded a light yellow viscous compound, 75% (w/w); IR ν ^{max} (neat): 1657 (chalcone), 1257, 1174, 1106, (ether), 1594, 1511, 1463, 1029, 985 (aromatics), 793 (furan moiety) cm^{-1}; ^{1}H, COSY- NMR (300 MHz, Acetone-d_6): δ 0.88 (6H, d, $J = 6.3$ Hz, H_3-13a, H_3-14a), 1.31 (3H, s, H_3-15a), 2.51 (1H, m, H-11a), 3.79 (1H, m, H_a-1a′), 4.11 (1H, m, H_b-1a′), 4.27 (2H, t, $J = 4.2$ Hz, H_2-2a′), 4.79 (1H, d, $J = 3.3$ Hz, αH-12a), 5.42 (1H, s, H-5a), 6.69 (1H, d, $J = 3.6$, H-4″), 7.02 (2H, d, $J = 8.7$ Hz, H-3′, H-5′), 7.52 (1H, d, $J = 3.0$, H-3″),7.51 (1H, d, $J = 15.6$, H-2), 7.72 (2H, d, $J = 3.6$ Hz, H-2″), 7.74 (2H, d, $J = 8.7$, H-2′, H-6′), 7.76 (1H, d, $J = 15.6$ Hz, H-3); ^{13}C, DEPT-NMR (75 MHz, Acetone-d_6): δ 12.83 (C-13a), 20.19 (C-14a), 24.66 (C-8a), 25.08 (C-2a), 25.78 (C-15a), 31.30 (C-11a), 34.98 (C-9a), 36.70 (C-3a), 37.60 (C-10a), 44.92 (C-7a), 53.02 (C-1a), 66.53 (C-1a′), 67.99 (C-2a′), 81.01 (C-6a, q), 87.93 (C-5a), 102.01 (C-12a), 103.89 (C-4a, q), 112.77 (C-3″), 115.51 (C-3′, C-5′), 117.74 (C-2″), 119.74 (C-2), 128.07 (C-1′, q), 130.82 (C-2′, C-6′), 143.21 (C-3), 147.39 (C-4″, q), 154.34 (C-1″, q), 161.64 (C-4′, q), 177.32 (C-1, q); HR-MS (*m*/*z*) $[M+H]^+$ (ESI^+) calcd for $C_{30}H_{37}O_8$, 525.2482; found, 525.2482. ESI-MS(positive): *m*/*z* 525 $[M+H]^+$, 547 $[M+Na]^+$, 563 $[M+K]^+$, molecular formula $C_{30}H_{36}O_8$.

*4.2.3.10. 1-[4′-{1a′-(12aβ-dihydroxyartemisinoxy)}-ethoxyphenyl]-3-(3″-hydroxyphenyl)-2-propen-1-one (**15**).* Elution of the column with *n*-hexane-ethyl acetate (70:40) yielded a viscous compound, 52% (w/w) yield, less polar than monomer **9**; IR ν ^{max}(neat): 3367 (OH), 1654 (chalcone), 1256, 1173, 1107 (ether), 1578, 1509, 1448, 1028, 984, (aromatics) cm^{-1}; ^{1}H, COSY- NMR (300 MHz, Acetone d_6): δ 0.81 (6H, d, J = 6.9 Hz, H_3-13a, H_3-14a), 1.17 (3H, s, H_3-15a), 2.42 (1H, m, H-11a), 3.70 (1H, m, H_a-1a′), 4.09 (1H, m, H_b-1a′), 4.19 (2H, d, J = 4.2 Hz, H_2-2a′), 4.71 (1H, d, J = 3.0 Hz, αH-12a), 5.36 (1H, s, H-5a), 6.96 (2H, d, J = 8.4 Hz, H-3′, H-5′), 7.03 (1H, dd, J = 8.10, 2.10 Hz, H-4″), 7.29 (1H, t, J = 7.80 Hz, H-5″), 7.47 (1H, brs, H-2″), 7.56 (1H, d, J = 15.3 Hz, H-2), 7.65(1H, d, J = 15.3 Hz, H-3), 7.72(3H, d, J = 8.4 Hz, H-2′, H-6′, H-6″), 7.90 (1H, brs, 4′-OH); ^{13}C, DEPT- NMR (75 MHz, Acetone-d_6): δ 12.78 (C-13a), 20.16 (C-14a), 24.64 (C-8a), 25.06 (C-2a), 25.73 (C-15a), 31.30 (C-11a), 34.96 (C-9a), 36.67 (C-3a), 37.58 (C-10a), 44.91 (C-7a), 53.02 (C-1a), 66.53 (C-1a′), 67.97 (C-2a′), 81.02 (C-6a, q), 87.93 (C-5a), 102.00 (C-12a), 103.89 (C-4a, q), 115.13 (C-2"), 115.48 (C-3′, C-5′), 120.06, 120.11, 120.23 (C-2,/C-4″,/C-6″), 128.30 (C-1′, q), 130.05 (C-5″), 130.82 (C-2′, C-6′), 140.44 (C-1″, q), 144.14 (C-3), 158.17 (C-4′, q), 161.58 (C-3″, q), 189.33 (C-1, q); HR-MS (m/z) $[M+H]^+$ (ESI^+) calcd for $C_{32}H_{39}O_8$, 551.2639; found, 551.2639. ESI-MS(positive): m/z 551 $[M+H]^+$, (Negative): 549 $[M-H]^-$, molecular formula $C_{32}H_{38}O_8$.

*4.2.3.11. 1-[4″-{1a′-(12aβ-dihydroxyartemisinoxy)}-ethoxyphenyl]-3-(1′-indolyl)-2-propen-1-one (**16**).* Elution of the column with *n*-hexane– ethyl acetate (70:20) yielded a viscous compound, 30% (w/w); IR ν ^{max} (neat): 3340, 2370 (NH), 1654 (chalcone), 1254, 1169, 1108, (ether), 1598, 1526, 1451, 1029, 983 (aromatics), 744 (pyrrole moiety) cm^{-1}; ^{1}H, COSY- NMR (300 MHz, Acetone-d_6): δ 0.76 (6H, m, H_3-13a, H_3-14a), 1.28 (3H, s, H_3-15a), 2.41 (1H, m, H-11a), 3.70 (1H, m, H_a-1a′), 3.92 (1H, m, H_b-1a′), 4.21 (2H, m, H_2-2a′), 4.67 (1H, brs, αH-12a), 5.31 (1H, s, H-5a), 6.97 (2H, d, J = 9.0 Hz, H-3″, H-5″), 7.12 (2H, dd, J = 6.6, 2.1 Hz, H-5′, H-6′), 7.40 (1H, d, J = 6.0, H-4′), 7.59 (1H, d, J = 15.6 Hz, H-2), 7.83 (1H, brs, H-2′, COSY correlation with NH), 7.96 (1H, d, J = 15.6 Hz, H-3), 7.99 (1H, m, H-7′), 8.02 (2H, d, J = 9.0 Hz, H-2″, H-6″), 10.80 (1H brs, NH); ^{13}C, DEPT-NMR (75 MHz, Acetone-d_6): δ 12.74 (C-13a), 20.10 (C-14a), 24.63 (C-8a), 25.05 (C-2a), 26.69 (C-15a), 31.30 (C-11a), 34.96 (C-9a), 36.66 (C-3a), 37.58 (C-10a), 44.93 (C-7a), 53.06 (C-1a), 66.53 (C-1a′), 67.99 (C-2a′), 81.00 (C-6a, q), 87.92 (C-5a), 102.01 (C-12a), 103.81 (C-4a, q), 112.64 (C-4′), 113.51 (C-1′, q), 114.76 (C-3″, C-5″), 120.10, 120.76, 121.48 (C-2,/C-6′,/C-7′), 123.16 (C-5′), 126.21 (C-3′, q), 130.71 (C-2″, C-6″), 131.20 (C-2″), 132.01 (C-1″, q), 138.08 (C-3), 138.70 (C-8′, q), 162.00 (C-4″, q), 185.50 (C-1, q); HR-MS (m/z) $[M+H]^+$ (ESI^+) calcd for $C_{34}H_{40}O_7N$, 574.2799; found, 574.2803. ESI-MS(positive): m/z 574 $[M+H]^+$. molecular formula $C_{34}H_{39}O_7N$.

*4.2.3.12. 1-[4"-{1a′-(12aβ-dihydroxyartemisinoxy)}-ethoxyphenyl]-3-(3′-ethoxy-4′-hydroxyphenyl)-2-propen-1-one (**17**).* Elution of the column with *n*-hexane-ethyl acetate (70:30) yielded a colorless viscous compound, 75% (w/w); IR ν ^{max} (neat): 3422 (OH), 1654 (chalcone), 1600, 1509, 1458, 1029, 984 (aromatics), 1560, 1261, 1168 (ether) cm^{-1}; ^{1}H, COSY- NMR (300 MHz, Acetone-d_6): δ 0.88 (6H, d, J = 6.6 Hz, H_3-13a, H_3-14a), 1.27 (3H, brs, H_3-15a), 1.37 (3H, t, J = 7.2 Hz, CH_3), 2.48 (1H, m, H-11a), 3.76 (1H, m, H_a-1a′), 4.10 (1H, m, H_b-1a′), 4.16 (2H, q, J = 7.5 Hz, OCH_2), 4.31 (2H, d, J = 3.9 Hz, H_2-2a′), 4.79 (1H, d, J = 3.3 Hz, αH-12a), 5.42 (1H, s, H-5a), 6.89 (1H, d, J = 8.1, H-5′), 7.07 (2H, d, J = 8.2 Hz, H-3″, H-5″), 7.28 (1H, dd, J = 8.1, 1.5 Hz, H-6′), 7.44 (1H, s, H-2′), 7.70 (2H, m, H-2, H-3), 8.11 (2H, d, J = 8.7 Hz, H-2″, H-6″); ^{13}C, DEPT- NMR (75 MHz, Acetone-d_6): δ 12.78 (C-13a), 14.56 (CH_3), 20.14 (C-14a), 24.64 (C-8a), 25.06 (C-2a), 25.73 (C-15a), 31.29 (C-11a), 34.95 (C-9a), 36.67 (C-3a), 37.58 (C-10a), 44.90 (C-7a), 53.02 (C-1a), 64.79 (OCH_2-), 66.49 (C-1a′), 68.03 (C-2a′), 81.00 (C-6a, q), 87.92 (C-5a), 102.01 (C-12a), 103.87 (C-4a, q), 112.50 (C-2′), 114.79 (C-3″, C-5″), 115.72 (C-5′), 119.43 (C-2), 123.75 (C-6′), 127.72 (C-1′, q), 130.98 (C-2″, C-6″), 131.95 (C-1″, q), 144.29 (C-3), 147.47 (C-4′, q), 149.91 (C-3′, q), 163.12 (C-4″, q), 187.64 (C-1, q); HR-MS (m/z) $[M+H]^+$ (ESI^+) calcd for $C_{34}H_{43}O_9$, 595.2901; found, 595.2900. ESI-MS(positive): m/z 595 $[M+H]^+$, 517 $[M+Na]^+$, 633 $[M+K]^+$, molecular formula $C_{34}H_{42}O_9$.

*4.2.3.13. 1-[4″-{1a′-(12aβ-dihydroxyartemisinoxy)}-ethoxyphenyl]-3-(2′, 3′, 4′-trimethoxy phenyl)-2-propen-1-one (**18**).* Elution of the column with *n*-hexane– ethyl acetate (70:30) yielded a yellow viscous compound, 75% (w/w); IR ν ^{max} (neat): 1656 (chalcone), 1601, 1494, 1463, 1030, 986 (aromatics), 1257, 1171 (ether) cm^{-1}; ^{1}H, COSY- NMR (300 MHz, Acetone d_6): δ 0.88 (6H, d, J = 6.3 Hz, H_3-13a, H_3-14a), 1.27 (3H, s, H_3-15a), 2.48 (1H, m, H-11a), 3.78 (1H, m, H_a-1_a′), 3.82, 3.89, 3.92, (3H each, s, 3xOCH_3), 4.12 (1H, m, H_b-1_a′), 4.31 (2H, d, J = 4.2 Hz, H_2-2a′), 4.79 (1H, d, J = 3.0 Hz, αH-12a), 5.42 (1H, s, H-5a), 6.86 (1H, d, J = 8.7 Hz, H-5′), 7.08 (2H, d, J = 8.7 Hz, H-3″, H-5″), 7.62 (1H, d, J = 8.7, H-6′), 7.75 (1H, d, J = 15.6 Hz, H-2), 7.98 (1H, d, J = 15.6 Hz, H-3), 8.11 (3H, d, J = 8.7 Hz, H-2″, H-6″); ^{13}C, DEPT-NMR (75 MHz, Acetone-d_6): δ 12.72 (C-13a), 20.21 (C-14a), 24.65 (C-8a), 25.07 (C-2a), 25.74 (C-15a), 31.29 (C-11a), 34.96 (C-9a), 36.67 (C-3a), 37.59 (C-10a), 44.89 (C-7a), 53.01 (C-1a), 56.01, 60.48, 61.37 (3xOCH_3), 66.49, 68.04 (C-1a′, C-2a′), 81.00 (C-6a, q), 87.91 (C-5a), 102.01 (C-12a), 103.88 (C-4a, q), 108.60 (C-5′), 114.85 (C-3″, C-5″), 120.94 (C-2), 122.22 (C-1′, q), 123.65 (C-6′), 131.90 (C-1″, q), 131.00 (C-2″, C-6″), 138.52 (C-3), 142.91 (C-3′, q), 153.98 (C-2′, q), 156.42 (C-4′, q), 163.17 (C-4″, q), 187.83 (C-1, q); HR-MS (m/z) $[M+H]^+$ (ESI^+) calcd for $C_{35}H_{45}O_{10}$, 625.3007; found, 625.3007. ESI-MS(positive): m/z 625 $[M+H]^+$, 647 $[M+Na]^+$, 663 $[M + K]^+$, molecular formula $C_{35}H_{44}O_{10}$.

*4.2.3.14. 1-[4″-{1a′-(12aβ-dihydroxyartemisinoxy)}-ethoxyphenyl]-1-(2′,4′-dimethoxyphenyl)-2-propen-1-one (**19**).* Elution of the column with *n*-hexane-ethyl acetate (70:25) yielded a viscous compound, 75% (w/w); IR ν ^{max} (neat): 1653 (chalcone), 1600, 1506, 1459, 1028, 985 (aromatics), 1260, 1168 (ether) cm^{-1}; ^{1}H, COSY-NMR (300 MHz, Acetone-d_6): δ 0.95 (3H, d, J = 9.3 Hz, H_3-13a), 0.91 (3H, d, J = 7.2 Hz, H_3-14a), 1.43 (3H, brs, H_3-15a), 2.61 (1H, m, H-11a), 3.83 (1H, m, H_a-1_a′), 3.86, 3.91 (3H each, s, 2xOCH_3), 3.97 (1H, m, H_b-1_a′), 4.19 (2H, m, H_2-2a′), 4.91 (1H, d, J = 3.3 Hz, αH-12a), 5.48 (1H, s, H-5a), 6.49 (1H, s, H-3′), 6.54 (1H, dd, J = 8.4, 2.1 Hz, H-5′), 6.97 (2H, d, J = 8.4 Hz, H-3″, H-5″), 7.54 (1H, d, J = 15.6 Hz, H-2), 7.57 (1H, d, J = 8.1, H-6′), 8.01 (2H, d, J = 8.4 Hz, H-2″, H-6″); ^{13}C, DEPT-NMR (75 MHz, Acetone-d_6): δ 13.33 (C-13a), 20.72 (C-14a), 24.81 (C-8a), 25.11 (C-2a), 26.56 (C-15a), 31.27 (C-11a), 35.00 (C-9a), 36.82 (C-3a), 37.86 (C-10a), 44.81 (C-7a), 52.95 (C-1a), 55.89, 55.96 (2×OCH_3), 66.79 (C-1a′), 67.90 (C-2a′), 81.51 (C-6a, q), 88.34 (C-5a), 98.88 (C-3′), 102.62 (C-12a), 104.53 (C-4a, q), 105.80 (C-5′), 114.67 (C-3″, C-5″), 117.70 (C-1′, q), 120.60 (C-2), 131.10 (C-2″, C-6″), 131.25 (C-6′), 132.17 (C-1″, q), 140.23 (C-3), 160.74 (C-4′, q), 162.83 (C-2′, q), 163.30 (C-4″, q), 189.90 (C-1, q); HR-MS (m/z) $[M+H]^+$ (ESI^+) calcd for $C_{34}H_{43}O_9$, 595.2901; found, 595.2902. ESI-MS(positive): m/z 595 $[M+H]^+$, 617$[M+Na]^+$, 633$[M+K]^+$, molecular formula $C_{34}H_{42}O_9$.

*4.2.3.15. 1-[4″-{1a′-(12aβ-dihydroxyartemisinoxy)}-ethoxyphenyl]-3-(3′-hydroxy-2′,4′-dimethoxyphenyl)-2-propen-1-one (**20**).* Elution of the column with *n*-hexane– ethyl acetate (70:30) yielded a viscous compound, 75% (w/w); IR ν ^{max} (neat): 3398 (OH), 1654 (chalcone), 1598, 1508, 1459 1027 (aromatics), 1279, 1125 (ether) cm^{-1}; ^{1}H, COSY- NMR (300 MHz, Acetone-d_6): δ 0.74 (3H, d, J = 7.2 Hz, H_3-13a), 0.76 (3H, d, J = 6.0 Hz, H_3-14a), 1.17 (3H, brs, H_3-15a), 2.31 (1H, m, H-11a), 3.57 (6H, s, 2xOCH_3), 3.84 (1H, m, H_a-1a′), 3.90 (1H, m, H_b-1a′), 4.08 (2H, m, H_2-2a′), 4.61 (1H, d, J = 3.3 Hz, αH-12a), 5.20 (1H, s, H-5a), 6.82 (2H, m, H-3″, H-5″), 7.03 (2H, brs, H-2′, H-6′), 7.55 (1H, d, J = 15.6 Hz, H-2), 7.67 (1H, d, J = 15.6 Hz, H-3),

7.93 (2H, d, J = 8.7 Hz, H-2″, H-6″), 9.18 (1H, m, 4′-OH); ^{13}C, DEPT-NMR (75 MHz, Acetone-d_6): δ 12.75 (C-13a), 20.25 (C-14a), 24.42 (C-8a), 25.05 (C-2a), 25.72 (C-15a), 31.31 (C-11a), 35.07 (C-9a), 36.69 (C-3a), 37.41 (C-10a), 45.00 (C-7a), 53.16 (C-1a), 56.16 (2xO<u>C</u>H$_3$), 68.58, 72.55 (C-1a', C-2a'), 81.06 (C-6a, q), 87.90 (C-5a), 102.23 (C-12a), 103.79 (C-4a, q), 106.55 (C-2′, C-6′), 115.66 (C-3″, C-5″), 121.42 (C-2), 130.75, 131.04 (C-1'/C-1″, q), 131.31 (C-2″, C-6″), 139.60 (C-4′, q), 143.75 (C-3), 154.08 (C-3′, C-5′, q), 162.23 (C-4″, q), 187.56 (C-1, q); HR-MS (m/z) [M+H]$^+$ (ESI$^+$) calcd for $C_{34}H_{43}O_{10}$, 611.2850; found, 611.2851. ESI-MS(positive): m/z 611 [M+H]$^+$, 633 [M+Na]$^+$, 649 [M+K]$^+$, molecular formula $C_{34}H_{42}O_{10}$.

*4.2.3.16. 1-[3″-{1a′-(12aβ-dihydroxyartemisinoxy)}-ethoxyphenyl]-3-[N-{1ba′-(12bβ-dihydroxyartemisinoxy)}-ethoxy-1H-1′-indolyl]-2-propen-1-one (**21**).* Elution of the column with *n*-hexane-ethyl acetate (70:40) yielded a viscous compound, 30% (w/w); IR ν ^{max} (neat): 2923, 2854, 1653 (chalcone), 1561, 1257, 1107 (ether), 1577, 1459, 1376, 1029, 984 (aromatics) cm^{-1}; ^{1}H, COSY- NMR (400 MHz, Acetone-d_6): δ 0.63 (6H, m, H$_3$-13a, H$_3$-13b), 0.76 (6H, m, H$_3$-14a, H$_3$-14b), 1.08, 1.17 (3H each, brs, H$_3$-15a, H$_3$-15b), 2.20, 2.41 (1H each, m, H-11a, H-11b), 3.62, 4.00, 4.19 (6H, m, H$_2$-1a′, H$_2$-1b′, H$_2$-2a′), 4.48 (2H each, m, H$_2$-2b′), 4.52, 4.67 (1H each, d, J = 2.7 and 2.4 Hz, αH-12a, αH-12b), 5.33 (2H, brs, H-5a, H-5b), 7.08 (2H, d, J = 8.12 Hz, H-4″), 7.16 (2H, m, H-5′, H-6′), 7.34 (1H, t, J = 7.40 Hz, H-5″), 7.48 (1H, d, J = 7.56 Hz, H-4′), 7.50 (1H, d, J = 2.56 Hz, H-2″), 7.54 (1H, d, J = 15.52 Hz, H-2), 7.59 (1H, d, J = 7.64 Hz, H-6″), 7.89 (1H, brs H-2′), 7.94 (1H, d, J = 6.64 Hz, H-7′), 7.97 (1H, d, J = 15.60 Hz, H-3); ^{13}C, DEPT and 2D-NMR (100 MHz, Acetone-d_6): δ 13.06, 13.22 (C-13a, C-13b), 20.51, 20.56 (C-14a, C-14b), 24.85, 25.06 (C-8a, C-8b), 25.19, 25.44 (C-2a, C-2b), 26.02, 26.12 (C-15a, C-15b), 31.47, 31.72 (C-11a, C-11b), 35.11, 35.36 (C-9a, C-9b), 36.91, 37.05 (C-3a, C-3b), 37.42, 37.98 (C-10a, C-10b), 44.92, 45.34 (C-7a, C-7b), 46.87 (C-2b′), 53.12, 53.44 (C-1a, C-1b), 66.29, 66.93, 68.40 (C-1a′, C-1b′, C-2a′), 81.11, 81.43 (C-6a, C-6b, q), 87.99, 88.32 (C-5a, C-5b), 101.84, 102.33 (C-12a, C-12b), 104.09, 104.26 (C-4a, C-4b, q), 111.66 (C-4′), 113.63 (C-1′, q), 114.67 (C-2″), 117.07 (C-2), 119.38 (C-4″), 121.28 (C-5′), 121.41 (C-6″), 122.15 (C-6′), 123.59 (C-7′), 127.30 (C-3′, q), 130.44 (C-5″), 135.82 (C-2′), 138.10 (C-8′, q), 138.99 (C-3), 141.60 (C-1″, q), 160.19 (C-3″, q), 189.21 (C-1, q); HR-MS (m/z) [M+H]$^+$ (ESI$^+$) calcd for $C_{51}H_{66}NO_2$, 884.4579; found, 884.4588. ESI-MS(positive): m/z 884 [M+H]$^+$, 906 [M+Na]$^+$, 922 [M+K]$^+$, molecular formula $C_{51}H_{65}NO_2$.

*4.2.3.17. 1-[4″-{1a′-(12aβ-dihydroxyartemisinoxy)}-ethoxy-3″-methylphenyl]-3-[4′-{1ba′-(12bβ-dihydroxyartemisinoxy)}-ethoxyphenyl]-2-propen-1-one (**22**).* Elution of the column with *n*-hexane-ethyl acetate (70:30) yielded a viscous compound, 75% (w/w); IR ν ^{max}: 2927, 2876, 1655 (chalcone), 1255, 1131 (ether), 1598, 1508, 1454, 1030, 985 (aromatics) cm^{-1}; ^{1}H, COSY- NMR (300 MHz, Acetone-d_6): δ 0.76 (12H, d, J = 7.2 Hz, H$_3$-13a, H$_3$-13b, H$_3$-14a, H$_3$-14b), 1.18 (6H, s, H$_3$-15a, H-15b), 2.14 (3H, s, C<u>H</u>$_3$), 2.35 (2H, m, H-11a, H-11b), 3.65 (2H, m, H$_a$-1a′, H$_a$-1b′), 4.01 (2H, m, H$_b$-1a′, H$_b$-1b′), 4.15 (4H, m, H$_2$-2a′, H$_2$-2b′), 4.66 (2H, d, J = 3.3 Hz, αH-12a, αH-12b), 5.30 (1H, s, H-5a, H-5b), 6.90 (1H, d, J = 7.8 Hz, H-5″), 6.95 (2H, d, J = 8.7 Hz, H-3′, H-5′), 7.61 (3H, m, H-2, H-3, H-2″), 7.63 (1H, d, J = 8.40 Hz, H-6″), 7.88 (2H, m, H-2′, H-6′); ^{13}C, DEPT- NMR (75 MHz, Acetone-d_6): δ 12.77 (C-13a, C-13b), 15.97 (C<u>H</u>$_3$), 20.11 (C-14a, C-14b), 24.62 (C-8a, C-8b), 25.05 (C-2a, C-2b), 25.71 (C-15a, C-15b), 31.29 (C-11a, C-11b), 34.96 (C-9a, C-9b), 36.65 (C-3a, C-3b), 37.58 (C-10a, C-10b), 44.89 (C-7a, C-7b), 52.98 (C-1a, C-1b), 66.53, 66.88 (C-1a′, C-1b′), 67.95, 68.07 (C-2a′C-2b′), 81.00 (C-6a, C-6b, q), 87.92 (C-5a, C-5b), 101.99, 102.19 (C-12a, C-12b), 103.88 (C-4a, C-4b, q), 111.04 (C-5″), 115.43 (C-3′, C-5′), 120.06 (C-2), 126.93 (C-3″, q), 128.50 (C-1′, C-1″, q), 128.87 (C-6″), 130.66 (C-2′, C-6′), 131.37 (C-2″), 143.18 (C-3), 161.33 (C-4′, C-4″, q), 187.23 (C-1, q); HR-MS (m/z) [M+H]$^+$ (ESI$^+$) calcd for $C_{50}H_{67}O_{13}$, 875.4576; found, 875.4578. ESI-MS(positive): m/z 875 [M+H]$^+$, 897 [M+Na]$^+$, 913 [M+K]$^+$, molecular formula $C_{50}H_{66}O_{13}$.

*4.2.3.18. 1-[4″-{1a′-(12aβ-dihydroxyartemisinoxy)-ethoxyphenyl]-3-[4′-{1b′-(12bβ-dihydroxyartemisinoxy)}-ethoxyphenyl]-2-propen-1-one (**23**).* Elution of the column with *n*-hexane− ethyl acetate (70:30) yielded a viscous compound, 75% (w/w); IR ν ^{max}: 1657 (chalcone), 1258, 1169, 1107 (ether), 1599, 1509, 1029, 985 (aromatics) cm^{-1}; ^{1}H COSY-, NMR (300 MHz, Acetone-d_6): δ 0.80 (12H, d, J = 7.5 Hz, H$_3$-13a, H$_3$-13b, H$_3$-14a, H$_3$-14b), 1.27 (6H, s, H$_3$-15a, H$_3$-15b), 2.48 (2H, m, H-11a, H-11b), 3.72 (2H, m, H$_a$-1a′, H$_a$-1b′), 4.20 (2H, m, H$_b$-1a′, H$_b$-1b′), 4.23 (4H, m, H$_2$-2a', H$_2$-2b'), 4.71 (2H, d, J = 3.0 Hz, αH-12a, αH-12b), 5.35 (2H, s, H-5a, H-5b), 6.95 (2H, d, J = 8.7 Hz, H-3′, H-5′), 7.00 (2H, d, J = 9.0 Hz, H-3″, H-5″), 7.69 (3H, m, H-2, H-2′, H-6′), 7.88 (2H, m, H-2, H-3), 8.05 (2H, d, J = 9.0 Hz, H-2″, H-6″); ^{13}C, DEPT- NMR (75 MHz, Acetone-d_6): δ 12.81 (C-13a, C-13b), 20.20 (C-14a, C-14b), 24.65 (C-8a, C-8b), 25.06 (C-2a, C-2b), 25.75 (C-15a, C-15b), 31.29 (C-11a, C-11b), 34.96 (C-9a, C-9b), 35.87 (C-10a, C-10b), 36.67 (C-3a, C3b), 44.90 (C-7a, C-7b), 53.00 (C-1a, C-1b), 66.54 (C-1a', C-1b'), 67.97 (C-2a'), 68.07 (C-2a'), 81.03 (C-6a, C-6b, q), 87.93 (C-5a, C-5b), 102.00 (C-12a, 12b), 103.93 (C-4a, C-4b, q), 114.86 (C-3′, C-5′), 115.46 (C-3″, C-5″), 119.92 (C-2), 128.43 (C-1′,q), 130.76 (C-2′, C-6′), 131.08 (C-2″, C-6″), 131.79 (C-1″), 143.54 (C-3), 161.44 (C-4′, q), 163.23 (C-4″, q), 187.72 (C-1, q); HR-MS (m/z) [M + H]$^+$ (ESI$^+$) calcd for $C_{49}H_{65}O_{13}$, 861.4419; found, 861.4423. ESI-MS(positive): m/z 861 [M+H]$^+$, 899 [M+K]$^+$, molecular formula $C_{49}H_{64}O_{13}$.

*4.2.3.19. 3-[4'-{1a'-(12aβ-dihydroxyartemisinoxy)}-ethoxy-3′, 5'-dimethoxyphenyl]-1-[4"-{1ba'-(12bβ-dihydroxyartemisinoxy)}-ethoxyphenyl]-2-propen-1-one (**24**).* Elution of the column with *n*-hexane-ethyl acetate (70:30) yielded a colorless solid, 75% (w/w); mp. 68 °C; IR ν ^{max} (neat): 1655 (chalcone), 1253, 1160, 1109 (ether), 1591, 1023, 982 (aromatics) cm^{-1}; ^{1}H, COSY- NMR (300 MHz, Acetone-d_6): δ 0.88 (12H, m, H$_3$-13a, H$_3$-13b, H$_3$-14a, H$_3$-14b), 1.16 (6H, s, H$_3$-15a, H$_3$-15b), 2.48 (2H, m, H-11a, H-11b), 3.75 (2H, m, H$_a$-1a', H$_a$-1b'), 3.91 (6H, brs, 2xOC<u>H</u>$_3$), 4.12 (2H, m, H$_b$-1a', H$_b$-1b'), 4.32 (2H, m, H$_2$-2a'), 4.33 (2H, m, H$_2$-2b'), 4.72, 4.81 (1H each, d, J = 3.0 Hz, αH-12a, αH-12b), 5.33 (2H, s, H-5a, H-5b), 7.08 (2H, d, J = 8.7 Hz, H-3″, H-5″), 7.16 (2H, brs, H-2′, H-6′), 7.70 (1H, d, J = 15.3 Hz, H-2), 7.79 (1H, d, J = 15.3 Hz, H-3), 8.12 (2H, d, J = 8.7 Hz, H-2″, H-6″); ^{13}C, DEPT- NMR (75 MHz, Acetone-d_6): δ 12.77 (C-13a, C-13b), 20.13 (C-14a), 20.28 (C-14b), 24.43 (C-8a), 24.64 (C-8b), 25.07 (C-2a, C-2b), 25.74 (C-15a, C-15b), 31.31 (C-11a, C-11b), 34.96 (C-9a), 35.09 (C-9b), 36.66 (C-3a), 36.71(C-3b), 37.42 (C-10a), 37.59 (C-10b), 44.90 (C-7a), 45.02 (C-7b), 53.09 (C-1a, C-1b), 56.18 (2xO<u>C</u>H$_3$), 66.48, 68.05 (C-1a′, C-1b′), 68.58, 72.56 (C-2a′, C-2b′), 80.98, 81.05 (C-6a, C-6b), 87.90 (C-5a, C-5b), 102.01, 102.23 (C-12a, C-12b), 103.78, 103.86 (C-4a, C-4b, q), 106.64 (C-2′, C-6′), 114.84 (C-3″, C-5″), 121.34 (C-2), 130.97, 131.73 (C-1′, C-1″, q), 131.08 (C-2″, C-6″), 144.05 (C-3), 154.10 (C-3′, C-4′ C-5′, q), 163.27 (C-4″, q), 187.60 (C-1, q); HR-MS (m/z) [M+H]$^+$ (ESI$^+$) calcd for $C_{51}H_{69}O_{15}$, 921.4630; found, 921.4630. ESI-MS(positive): m/z 921 [M+H]$^+$, 943 [M+Na]$^+$, 959 [M+K]$^+$, molecular formula $C_{51}H_{68}O_{15}$.

*4.2.3.20. 7-{1a′-(12aβ-dihydroxyartemisinoxy)}-ethoxy-4′-{2a′-(12bβ-dihydroxyartemisinoxy)]-ethoxyflavone (**25**).* Elution of the column with *n*-hexane-ethyl acetate (70:30) yielded a colorless solid, 55% (w/w), mp. 64 °C; IR ν ^{max}: 1457, 1368, 1029, 985 (aromatics), 1684 (αβ unsaturated CO) cm^{-1}; ^{1}H, COSY- NMR (400 MHz, Acetone-d_6): δ 0.90 (12H, m, H$_3$-13a, H$_3$-13b, H$_3$-14a, H$_3$-14b), 1.31 (6H, s, H$_3$-15a, H$_3$-15b), 2.52 (2H, m, H-11a, H-11b), 3.79 (2H, m, H$_a$-1a′, H$_a$-1b′), 4.12 (2H, m, H$_b$-1a′, H$_b$-1b′), 4.32 (4H, m, H$_2$-2a′, H$_2$-2b′), 4.79 (2H, d, J = 2.92 Hz, αH-12a, αH-12b), 5.42, 5.44 (1H each, s,

H-5a, H-5b), 6.51 (1H, d, $J = 2.4$ Hz, H-8), 6.58 (1H, m, H-6), 7.06 (2H, d, $J = 8.68$, H-3′, H-5′), 7.84 (2H, d, 8.72 Hz, H-2′, H-6′), 7.86 (1H, s, H-3), 8.20 (1H, d, $J = 8.96$, H-5); ^{13}C, DEPT- NMR (100 MHz, Acetone-d_6): δ 12.36, 12.37 (C-13a, C-13b), 19.47, 19.74 (C-14a, C-14b), 24.23 (C-8a, C-8b), 24.66 (C-2a, C-2b), 25.30 (C-15a, C-15b), 31.72 (C-11a, C-11b), 34.56 (C-9a, C-9b), 37.19 (C-10a, C-10b), 36.25 (C-3a, C3b), 44.33 (C-7a, C-7b), 52.62 (C-1a, C-1b), 66.82, 66.93 (C-1a′, C-1b′), 68.42, 68.57 (C-2a′, C-2b′), 81.44 (C-6a, C-6b, q), 88.34 (C-5a, C-5b), 102.40, 102.49 (C-12a, 12b), 104.31 (C-4a, C-4b, q), 115.47, 115.94 (C-3′, C-5′), 118.97 (C-6), 128.57 (C-5), 128.80 (C-4a*, q), 131.64, 132.87 (C-2′, C-6′), 132.36 (C-1′, q), 145.09 (C-3), 162.29 (C-4′), 164.37 (C-8a, q), 166.50 (C-7, q), 167.53 (C-2, q), 192.95 (C-4, q); HR-MS (m/z) $[M+H]^+$ (ESI^+) calcd for $C_{49}H_{63}O_{14}$, 875.4212; found, 875.4213. ESI-MS(positive): m/z 875 $[M+H]^+$, molecular formula $C_{49}H_{62}O_{14}$.

4.3. In vitro cytotoxic studies of artemisinin derivatives

4.3.1. Cell culture, growth conditions and treatment

Human leukemia cell line HL-60, pancreatic cancer cell line Mia PaCa-2, prostate cancer cell line PC-3, hepatic cancer cell line HEP-G2 and colon cancer cell line LS180 and VERO cells were obtained from ECACC, England. Cells were grown in RPMI-1640/DMEM/MEM/McCoy's medium containing 10% FCS and 1X antibiotic antimycotic solution from invitrogen. Cells were grown in CO_2 incubator (Thermocon Electron Corporation, USA) at 37 °C with 95% humidity and 5% CO_2 gas environment. Test compounds used in cells treatment were dissolved in DMSO while the untreated cultures received only the vehicle (DMSO, <0.2%, v/v).

4.3.2. Chemicals

Propidium iodide, Dihydrorhodamine 123 (DHR123), DNase-free RNase, 3-(4,5,-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33342, ethanol were purchased from M/s Sigma chemicals Co., India. Fetal bovine serum was obtained from M/s GIBCO Invitrogen Corporation, USA.

4.3.3. MTT cell proliferation assay

In vitro cytotoxic effects of test compounds were determined by MTT dye, which is converted into formazan crystals by mitochondria of healthy cells. Cells (1.5×10^4/200 μl media for suspension cell line and 70–75% confluent for adherent cell lines) were cultured in 96-well culture plates and treated with various concentrations of compounds for 48 h. MTT dye was added 3 h prior to experiment termination. The MTT formazan crystals formed were dissolved in 150 μL of DMSO and OD measured at 570 nm (reference wave length 620 nm). % growth inhibition was calculated by comparing the absorbance of treated verses untreated cells. Selectivity index (SI) of each compound with different cell lines was calculated as follow, SI= IC_{50} of VERO cell/IC_{50} of compound on different tested cell line. Doxorubicin was used as a reference drug.

4.3.4. Cell cycle analysis

HL-60 cells (0.5×10^6 cells/ml/12well plate) were treated with compounds dihydroarteminsin (DHA), **8** and **15** at 0.2, 0.5, 1 and 5 μM concentrations for 24 h time period. Cells were collected at 400×*g* for 5 min in 5 ml polystyrene tubes. Cell pellets were washed twice with PBS and fixed in 70% ethanol at 4 °C overnight. Cells were collected next day, washed with PBS and resuspended in 250 μl of PBS. Cells were incubated with RNaseA (200 μg/ml) at 37 °C for 1.30 h and then after stained with PI (10 μg/ml) for 20 min. The cells were acquired for PI-DNA fluorescence by flow cytometer and data were collected in list mode on 10,000 events for FL2-A vs. FL2-W. Apoptotic population were analyzed by ModFit software from Verity software house.

4.3.5. Mitochondrial potential loss

Mitochondrial membrane potential (Ψm) was measured by using dihydrorhodamine 123 (Rh123) dye which is used to monitor membrane potential of mitochondria. Cells (0.5×10^6 cells/ml/12well plate) were treated with dihydroarteminsin (DHA), **8** and **15** at 0.2, 0.5, 1 and 5 μM for 24 h. Cells were stained with the dye (200 nM concentration) 30 min prior to experiment termination. Cells were collected at 400×g, washed with PBS and analyzed on FL-1 channel of flow cytometer (BD FACS Calibur). The decrease in FL-1 fluorescence indicates the loss of mitochondrial membrane potential in cells.

4.3.6. Hoechst staining and phase contrast microscopy

HL-60 cells (0.5×10^6 cells/ml/12well plate) were seeded in culture plates and treated with compounds dihydroarteminsin (DHA), **8** and **15** at 0.2, 0.5, 1 and 5 μM concentrations for 24 h. Cells were collected at 400×g, washed with PBS twice and fixed in 1 ml of fixing solution (cold acetic acid: methanol (1:3), v/v) for 1 h on ice. Cells were centrifuged again at 400 ×g and resuspended in 100 μl of fixing solution. Cells were spreaded on a clean cold slide and dried overnight at room temperature. Next day, hoechst 33258 (5 μg/ml in 0.01 M citric acid and 0.45 M disodium phosphate containing 0.05% Tween 20) were poured on the slides for 30 min. After incubation, slides were washed with PBS twice and while wet, 30 μl of mounting fluid containing glycerol: PBS at equal ratio were poured over the slides. The slides was covered with glass cover slip and sealed with nail polish. Photographs were taken under microscope at 20× by using Olympus IX70 microscope. For phase contrast microscopy, treated and control cells were photographed under microscope after treatment.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.06.035.

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