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**Flaviano Melo Ottoni**

UFMG: Universidade Federal de Minas Gerais

**Lucas Bonfim Marques**

UFMG: Universidade Federal de Minas Gerais

**Juliana Martins Ribeiro**

UFMG: Universidade Federal de Minas Gerais

**Lucas Lopardi Franco**

UFMG: Universidade Federal de Minas Gerais

**José Dias Souza Filho**

UFMG: Universidade Federal de Minas Gerais

**Rodrigo Maia Pádua**

UFMG: Universidade Federal de Minas Gerais

**Elaine Maria Souza-Fagundes**

UFMG: Universidade Federal de Minas Gerais

**Ricardo José Alves** (✉ [ricardodylan@farmacia.ufmg.br](mailto:ricardodylan@farmacia.ufmg.br))

Federal University of Minas Gerais State <https://orcid.org/0000-0002-1666-4421>

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## Research Article

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## Synthesis of lapachol-based glycosides and glycosyl triazoles with antiproliferative activity against several cancer cell lines

Flaviano M. Ottoni<sup>1</sup> . Lucas B. Marques<sup>2</sup> . Juliana M. Ribeiro<sup>2</sup> . Lucas L. Franco<sup>3</sup> . José D. S. Filho<sup>4</sup> . Rodrigo Maia de Pádua<sup>1</sup> . Elaine M. Souza-Fagundes<sup>2</sup> . Ricardo J. Alves<sup>1</sup>

✉ Ricardo J. Alves

[ricardodylan@farmacia.ufmg.br](mailto:ricardodylan@farmacia.ufmg.br)

<sup>1</sup>Departamento de Produtos Farmacêuticos, Faculdade de Farmácia, Universidade Federal de Minas Gerais (UFMG), Avenida Antônio Carlos, 6627, Belo Horizonte, MG 31.270-901, Brazil.

<sup>2</sup>Departamento de Biofísica e Fisiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (UFMG), Avenida Antônio Carlos, 6627, Belo Horizonte, MG 31.270-901, Brazil.

<sup>3</sup>Laboratório de Pesquisa em Química Farmacêutica (LQFar), Faculdade de Ciências Farmacêuticas, Universidade Federal de Alfenas (UNIFAL), Alfenas, MG 37130-001, Brazil

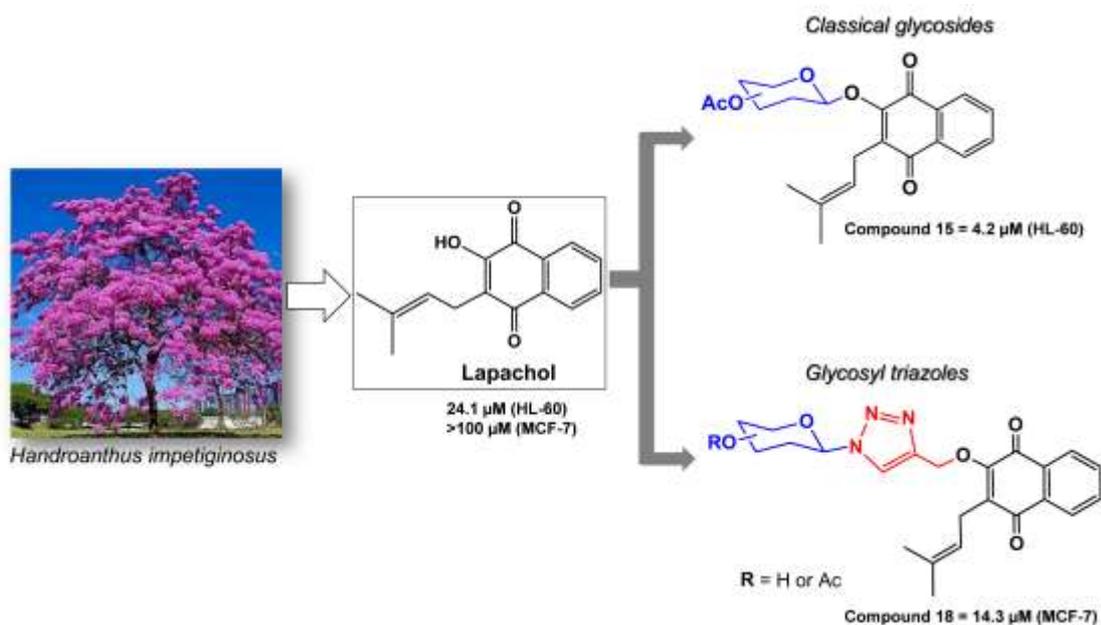
<sup>4</sup>Departamento de Química, Instituto de Ciências Exatas, Universidade Federal de Minas Gerais (UFMG), Avenida Antônio Carlos, 6627, Belo Horizonte, MG 31.270-901, Brazil.

**ABSTRACT** Lapachol (**1**), a natural naphthoquinone, presents several biological activities including antitumor activity, used as anticancer adjuvant whose use was abandoned because of adverse effects. Herein, we reported the synthesis and cytotoxicity evaluation against cancer cell lines of a series of *O*-glycosides and glycosyl triazoles derived from lapachol. In addition to the determination of IC<sub>50</sub>, the DNA fragmentation and clonogenicity were also evaluated. The glycoside derived from D-glucose (**5**) was far more active than lapachol (**1**) and more active in tumor cell lines HL60, Jurkat, THP-1 and MDA-MB-231 than to the non-tumoral PBMC cell line, indicating an improvement in activity and selectivity as compared with lapachol (**1**). Compound **5** and the glycosides derived from D-galactose (**14**), D-*N*-acetylglucosamine (**15**) and L-fucose (**16**) showed good results in the DNA fragmentation and clonogenicity assays in the studies of subdiploid DNA content, indicating a pro-apoptotic potential and a good antiproliferative activity of these glycosides.

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<sup>1</sup>\* Corresponding author. Tel.: +55 (31) 3409-6955; fax: +55-(31)-3409-6935; e-mail: [ricardodylan@farmacia.ufmg.br](mailto:ricardodylan@farmacia.ufmg.br)

## Graphical abstract



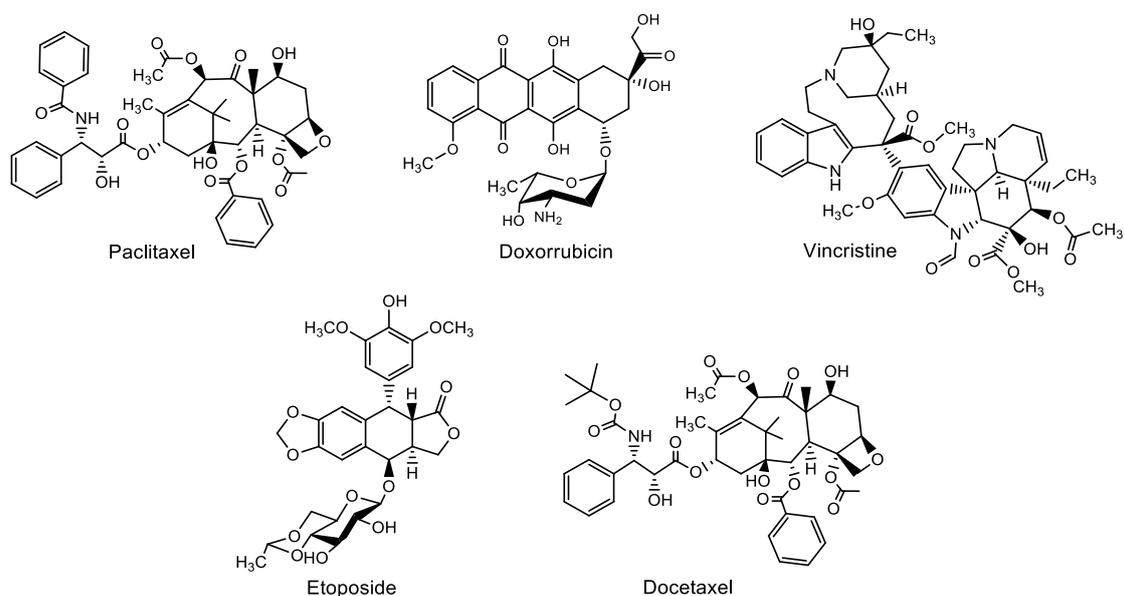
## Keywords

Lapachol, glycosides, glycosyl triazoles, DNA fragmentation, clonogenicity, antiproliferative activity

## 1. Introduction

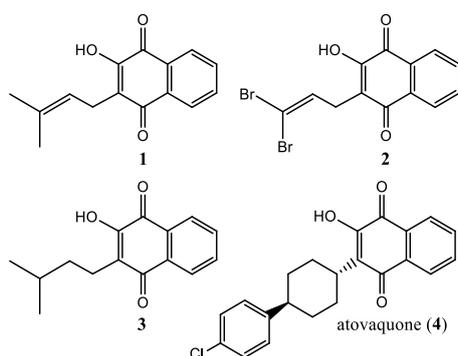
Cancer is one of the principal causes of death worldwide and its incidence is expected to increase in the next years. According to WHO, about 30 million cases of cancer are expected to occur until 2040, with almost 50% taking place in developing countries [1]. Despite the great efforts in prevention and treatment of cancer there is a continuous need for new options. The lack of selective action towards the cancer cells of most current existing anticancer drugs results in toxicity to host tissues. Thus, the search for new potent and safer drugs of synthetic and natural origin is being pursued by several groups around the world.

Natural (paclitaxel, doxorubicin, vincristine) and semi synthetic compounds (etoposide and docetaxel) are among the most important anticancer drugs (Fig. 1). Etoposide and docetaxel are good examples of how synthetic modification can contribute to improve the activity of bioactive natural compounds [2, 3].



**Fig. 1.** Anticancer agents of natural and semi synthetic origin.

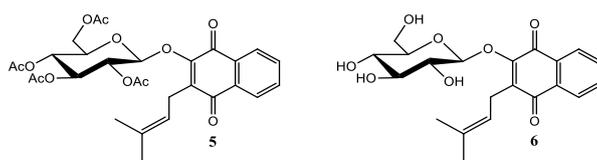
Lapachol (**1**), 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthalenedione is a natural 1,4-naphthoquinone isolated from plants of the Bignoniaceae family, mainly *Handroanthus impetiginosus*. It presents several biological activities including antitumor activity [4, 5]. This compound has been used as coadjuvant in the chemotherapy of certain tumors but its use was abandoned because of adverse effects, mainly related to blood clotting.[5, 6] Some synthetic routes were established to get lapachol (**1**), firstly by Fieser [7], being obtained in low yields. Recently, lapachol was synthesized from lawsone in better yields [8]. Several derivatives of **1** were prepared with antifungal, antibacterial, antiviral and antitumor activity [5]. Eyong *et al.* described the synthesis of atovaquone, which was approved for treatment of pneumonia (*Pneumocystis pneumonia*), toxoplasmosis and malaria [4, 9]. Atovaquone is a naphthoquinone as well as lapachol and studies carried out in the last years have shown that atovaquone has a potent antitumor activity [10]. The chemical structure of **1** and some of its derivatives with antitumor activity are shown below (Fig. 2).



**Fig. 2.** Chemical structure of **1** and some derivatives showing antitumor activity [4, 5].

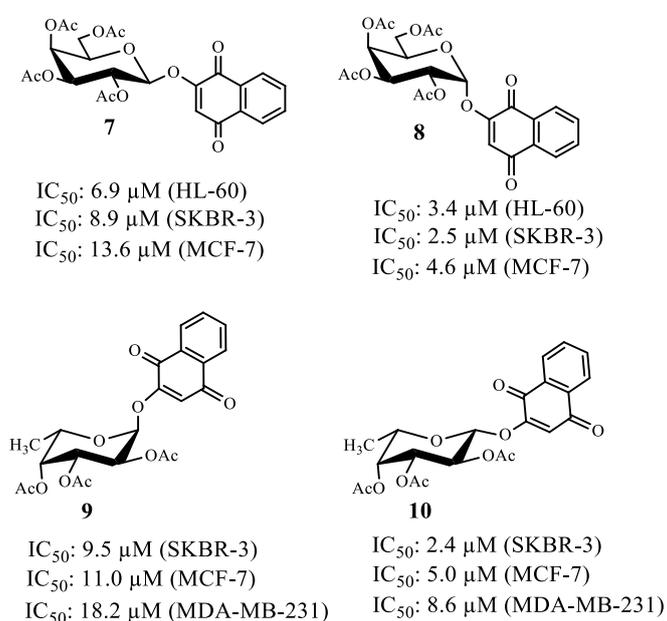
The quinones are able to inhibit the mitochondrial oxidation and phosphorylation, as well to inhibit the enzyme succinate oxidase [11] which plays an important role in the citric acid cycle and the electron transport chain. Other mechanisms seem to be related to the intercalation of the naphthoquinones between the DNA base pairs [5] and inhibition of topoisomerases [12]. The main mechanism of action is related to the formation of reactive oxygen species (ROS), through semiquinone radicals. Both cause damage to cell macro molecules and consequently cell death [12].

The major problem for the clinical use of **1** is its low bioavailability, due to low water solubility, which implicated in the use of large doses for attaining plasmatic levels, causing severe side effects. The first attempt to enhance water solubility of lapachol was reported by Linardi and co-workers who described the synthesis of the  $\beta$ -D-glucoside of **1** (compound **6**) and the corresponding peracetylated derivative **5** (Fig. 3) [13]. These two compounds were evaluated *in vivo* in mice bearing P-388 lymphocytic leukemia. The peracetylated glucoside **5** was active, enhancing the lifespan of mice by 80%, while the deacetylated derivative was inactive. According to the authors, the peracetylated glucoside was possibly acting as prodrug that could be absorbed by the cancer cells due to its lipophilic character. The unprotected derivative **6**, being more hydrophilic, was possibly unable to cross the cell membranes being, therefore, inactive.[13]



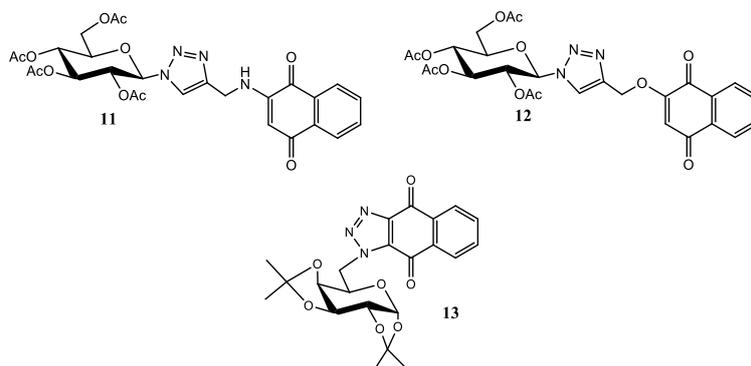
**Fig. 3.** Chemical structures of the peracetylated  $\beta$ -D-glucoside (**5**) and  $\beta$ -D-glucoside (**6**) of lapachol.

Several anticancer drugs possess a carbohydrate moiety in their structures, as shown in Fig. 1 for etoposide and doxorubicin. The work of Linardi and co-workers [13] showed that the attachment of a glucosyl moiety to lapachol can be a good approach to obtain new anticancer compounds. Glycosidic derivatives of lawsone, another naphthoquinone, has been obtained and assayed for antitumor activity. The glycosides were cytotoxic against HL-60 (acute promyelocytic leukemia), SKBR-3, MCF-7 and MDA-MB-231 (breast cancer) cells indicating that the variation of the carbohydrate moiety and the anomer type ( $\alpha$  or  $\beta$ -glycoside) influence the cytotoxicity [14, 15]. Some these lawsone glycosides with antitumor activity are shown in Fig. 4.



**Fig. 4.** Chemical structures of synthetic glycosides derived from lawsone active against cancer cells.

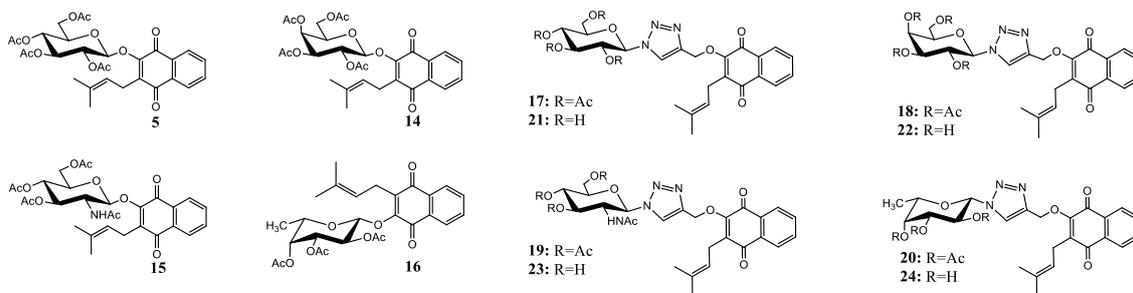
Besides classical glycosides, obtained by direct glycosylation, one strategy widely used to link a carbohydrate moiety to a natural or synthetic compound is the Cu(I)-catalyzed cycloaddition reaction between an alkyne derivative of the compound with a glycosyl azide, to get glycosyl triazoles [16]. Based on this, several glycosyl triazoles derived from naphthoquinones with antitumor activity are described in the literature (Fig. 5) [17–19].



**Fig. 5.** Chemical structures of synthetic glycosyl triazoles derived from naphthoquinones.

Recently we described the synthesis and cytotoxic evaluation against HL-60 human leukemia cells of lapachol glycosides **5** and **15**. These compounds showed low  $IC_{50}$  values, *circa* 5.0  $\mu$ M. The mechanism of cytotoxic seems to involve the activation apoptosis signaling pathways, such as the DNA fragmentation, chromatin condensation and decrease of the mitochondrial transmembrane potential [20].

In the present work we describe the synthesis and cytotoxicity evaluation against cancer cell lines of a series of *O*-glycosides and glycosyl triazoles derived from lapachol. The structures of the synthesized compounds are shown in Fig. 6.



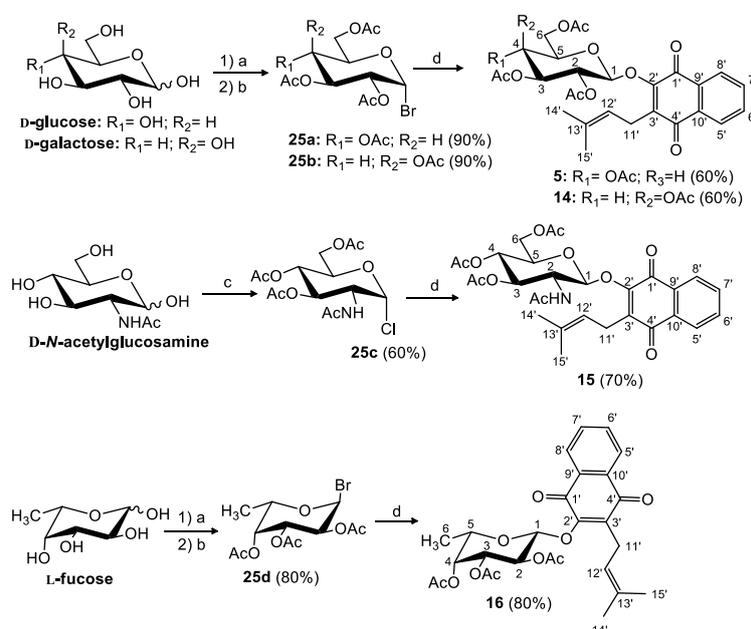
**Fig. 6.** Chemical structures of the glycosyl triazoles derived from lapachol synthesized in this work.

The presence and orientation of groups (OH and NHAc) that can modulate the physico-chemical properties of the compounds was considered, taking into account that the parent carbohydrates have different solubility and that the *O*-acetyl groups confer lipophilic properties to the peracetylated derivatives. We also considered the presence of specific carbohydrate transporters in the cell surface [21], that should facilitate the transport of the deacetylated glycosides across the cancer cell membrane.

## 2 Results and discussion

### 2.1 Chemistry

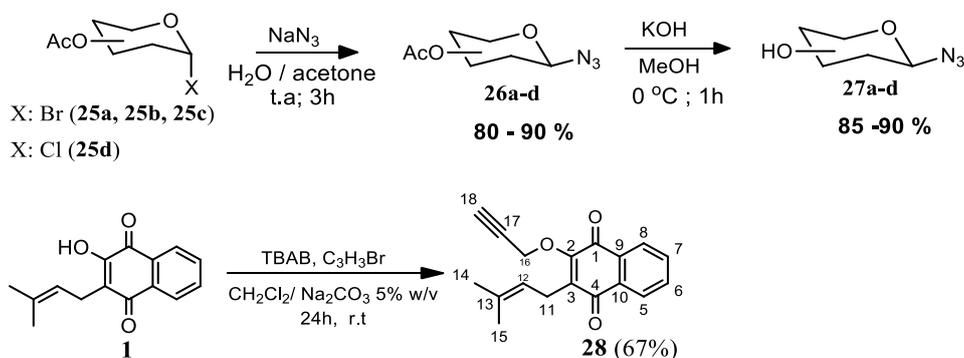
The classic glycosides of lapachol (**1**) were obtained by its reaction with the glycosyl halides [22–24] under phase transfer catalysis (PTC) conditions: 10% w/v sodium carbonate aqueous solution, dichloromethane and tetra-*n*-butyl bromide (TBAB) as phase transfer catalyst [25–27]. As glycosyl halides were used peracetylated glycosyl bromides from D-glucose, D-galactose [23] and L-fucose [24] and the peracetylated D-*N*-acetylglucosaminyl chloride [22]. The compounds were synthesized according to the synthetic scheme shown below (Scheme 1).



**Scheme 1.** Reagents and conditions (a) Ac<sub>2</sub>O, AcONa, 100 °C, 5 h, [90%]; (b) HBr/AcOH, CH<sub>2</sub>Cl<sub>2</sub>, r.t, 6 h, [90-95%]; (c) CH<sub>3</sub>COCl, r.t, 48 h, [50%]; (d) Lapachol, CH<sub>2</sub>Cl<sub>2</sub>, Na<sub>2</sub>CO<sub>3</sub> 10 % p/v (1:1), *n*-Bu<sub>4</sub>NBr, r.t, 8 h, [60-80%] .r.t. = room temperature; yields = [ ].

The peracetylated glycosides from D-glucose, D-galactose and L-fucose were prepared using sodium acetate (AcONa) and acetic anhydride (Ac<sub>2</sub>O) as solvent at 100 °C. The treatment of peracetylated carbohydrates with HBr/AcOH solution at room temperature furnished the corresponding glycosyl bromide [23, 24]. The peracetylated glycosyl halide derived from D-*N*-acetylglucosamine was obtained reacting D-*N*-acetylglucosamine with acetyl chloride at room temperature for 48 h [22]. Finally, in the glycosylation step the reaction of lapachol (**1**) with each of the glycosyl halides (**25a-d**) afforded the corresponding peracetylated lapachol glycosides **5** and **14-16** in 60-80% yields.

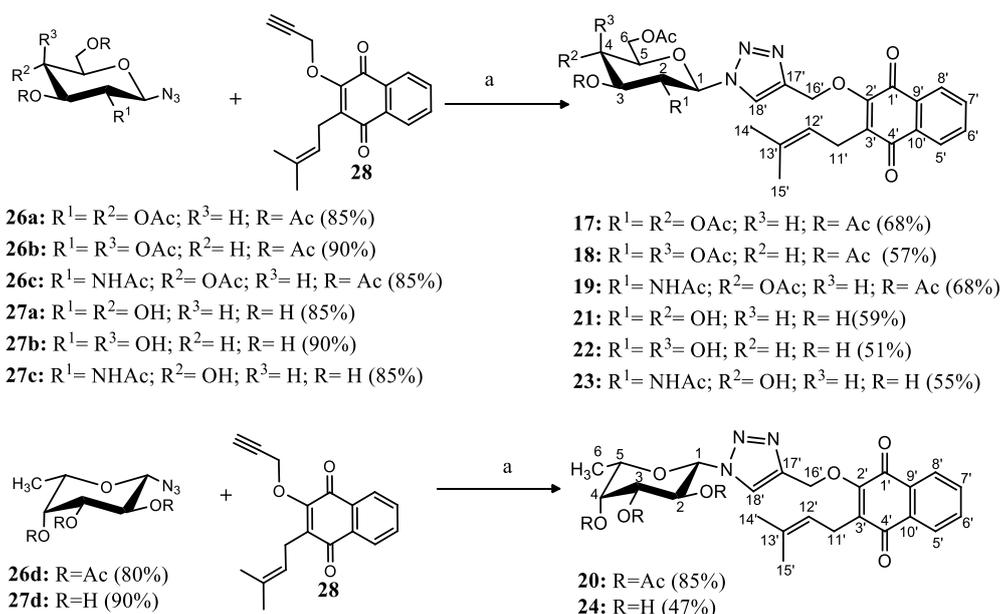
The glycosyl triazoles of **1** were synthesized using “click” chemistry [28–30] namely, the reaction of the 2-*O*-propargyllapachol (**28**) with glycosyl azides (**26** and **27**) [31–34], prepared according to the synthetic scheme shown below (Scheme 1).



**Scheme 2.** Reaction conditions for the obtention of 2-*O*-propargyllapachol and the glycosyl azides.

Treatment of lapachol with propargyl bromide under phase-transfer conditions [25–27] furnished the corresponding 2-*O*-propargyllapachol (**28**) in 67 % yield. The glycosyl azides **26a-d** were obtained from the corresponding peracetylated glycosyl halides (**25a-d**) according to literature procedures, namely, reaction with NaN<sub>3</sub> in acetone/H<sub>2</sub>O at room temperature [35]. Deacetylation of **26a-d** under standard conditions (KOH/MeOH at 0 °C) [33, 34] furnished the corresponding unprotected glycosyl azides **27a-d** in good yields (Scheme 2).

Finally, Cu(I)-catalyzed cycloaddition reaction of **28** with each of the glycosyl azides (**26a-d** and **27a-d**) afforded the corresponding peracetylated (**17-20**) and deacetylated (**21-24**) glycosyl triazoles of lapachol yields ranging from 47% to 85% (Scheme 3).



**Scheme 3.** (a) Cu(OAc)<sub>2</sub>·H<sub>2</sub>O, sodium ascorbate 60% mol, THF:H<sub>2</sub>O (1:1), r.t., 2-5h, [47-85%]. r.t. = room temperature; yields = [ ].

The compounds were characterized by IR, NMR and ESI-MS spectroscopy. The infrared spectra of the peracetylated glycosides of lapachol **5** and **14-16** showed bands at 3039-2858 cm<sup>-1</sup> (C-H alkane and aromatic), 1748-1742 cm<sup>-1</sup> (C=O, ester) and 1224-1213 cm<sup>-1</sup> (C-O, ester). In the infrared spectrum of compound **16** one observes bands at 3308 cm<sup>-1</sup> (stretching N-H), 1666 cm<sup>-1</sup> (C=O amide) and 1539 cm<sup>-1</sup> (bending N-H) correspondent to acetamido group in C-2. The <sup>1</sup>H NMR spectra of these compounds showed signals at δ<sub>H</sub> 1.65-1.83 ppm (methyl groups at C-14' and C-15' of the lapachol moiety), δ<sub>H</sub> 1.94-2.20 ppm (CH<sub>3</sub>C=O), δ<sub>H</sub> 3.30-3.45 ppm (methylene hydrogens at C-11' of the lapachol), δ<sub>H</sub> 3.66-5.83 ppm (pyranosidic protons), δ<sub>H</sub> 5.09-5.13 ppm (olefinic hydrogens H-12' of the isoprenyl side chain) and 7.69-8.10 ppm (aromatic hydrogens of lapachol). In the <sup>1</sup>H NMR spectrum of compound **15** (D-N-acetylglucosamine derivative) the signal corresponding at N-H resonates at δ<sub>H</sub> 6.35 ppm (*d*, *J*=8.8 Hz; 1H, N-H acetamide) and the signal of H-6 of the compound **16** (L-fucose derivative) is recorded at as a duplet at δ<sub>H</sub> 1.14 ppm (*d*, *J*=6.4 Hz; 1H, H-6L-fucose). All lapachol glycosides correspond to β anomer as confirmed by <sup>1</sup>H NMR spectrometry, wherein one observes signals at δ<sub>H</sub> 5.36 and δ<sub>H</sub> 5.84, corresponding at H-1 (anomeric hydrogen), with *J*-coupling values around 8.0 Hz. According to Karplus's rule coupling between vicinal hydrogens with *J*-coupling values ranging 8.0 to 10.0 Hz correspond *trans*-diaxial coupling compatible with β-type glycosides [36]. These structural features were confirmed by the <sup>13</sup>C NMR spectra of these compounds, which showed signals at δ<sub>C</sub> 17.7-18.3 ppm (CH<sub>3</sub>, C-14'), δ<sub>C</sub> 20.3-21.1 ppm (CH<sub>3</sub>,

ester),  $\delta_C$  23.2-23.7 ppm (CH<sub>2</sub>, C-11'),  $\delta_C$  25.5-26.1 ppm (CH<sub>3</sub>, C-15'),  $\delta_C$  54.3-73.3 ppm (CH, C-2 to C-5),  $\delta_C$  99.3-100.4 ppm (CH, C-1'),  $\delta_C$  119.2-119.6 ppm (CH,C-12'),  $\delta_C$  125.8-134.5 ppm (CH,C-5' at C-10'),  $\delta_C$  137.5-139.1 ppm (CH,C-3'),  $\delta_C$  152.6-153.2 ppm (CH,C-2'),  $\delta_C$  169.5-171.0 ppm (C=O, ester) and,  $\delta_C$  180.4-185.2 ppm (C=O, quinone). In the <sup>13</sup>C NMR spectrum of **15** one observes signals at  $\delta_C$  23.4 ppm (CH<sub>3</sub>, amide) and at  $\delta_C$  168.3 ppm (C=O, amide). For compound **16** one observes a signal at  $\delta_C$  15.6 ppm (CH<sub>3</sub>, C-6). The  $\beta$ -configuration at the anomeric carbon of glycosides **5** and **14-16** was confirmed by the resonance signals at  $\delta_C$  99.3-100.4 (CH, C-1').

The infrared spectrum of the 2-*O*-propargyllapachol (**28**) showed absorption bands at 3355-3279 cm<sup>-1</sup> (C-H alkyne), 2964-2953 cm<sup>-1</sup> (C-H sp<sup>3</sup>), 2124 cm<sup>-1</sup> (C-C alkyne), 1660-1640 cm<sup>-1</sup> (C=C olefin), 1610-1593 cm<sup>-1</sup> (C=C aromatic), 1186 and 1047 cm<sup>-1</sup> (C-O). The <sup>1</sup>H NMR spectrum of **28** showed signals assigned to the alkyne terminal hydrogen H-18 at  $\delta_H$  2.51 ppm (*d*, *J*=2.0 Hz) and the methylene hydrogens H-16 at  $\delta_H$  5.14 ppm (*d*, *J*=2.4 Hz), both as a doublet. In the <sup>13</sup>C NMR spectrum of **28** one observes signals corresponding to the propargyl group at  $\delta_C$  60.3 ppm (methylene carbon C-16),  $\delta_C$  76.4 ppm and  $\delta_C$  78.4 ppm (alkyne carbons C-17 and C-18).

The infrared spectra of the peracetylated glycosyl triazoles of lapachol **17-20** were similar to those of the lapachol glycosides **5** and **14-16** (N-H, C-H, C=O, C=C and C-O stretching and N-H bend). The <sup>1</sup>H NMR spectra of the glycosyl triazoles **17-20** showed a singlet at  $\delta_H$  7.98-8.55 ppm corresponding to the hydrogen of the triazole ring (H-18). As expected, all glycosyl triazoles **17-24** are of  $\beta$  configuration, the same as the glycosyl azides, which were obtained by S<sub>N</sub>2 type nucleophilic substitution from glycosyl halides, which present  $\alpha$  configuration already stabilized by the anomeric effect [37]. For example, in the <sup>1</sup>H NMR spectrum of compound **17** one observes a signal at  $\delta_H$  6.37 corresponding to H-1, with *J*-coupling value (*J*<sup>3</sup>) around 8.0 Hz. As discussed previously these vicinal coupling constant values correspond to *trans*-diaxial coupling and confirm the  $\beta$  configuration of glycosyl triazoles of lapachol (**17-24**) [36].

In the <sup>13</sup>C NMR spectrum of these compounds the anomeric carbon (C-1) signals are observed at 84.3-86.5 ppm and triazole ring carbon (C-18) at  $\delta_C$  122.0-133.8 ppm. The signal related to the other carbon of the triazole ring (C-17) was observed only in the <sup>13</sup>C NMR spectra of compounds **17** (143.7 ppm) and **19** (143.3 ppm), probably due to the relaxation time of such carbon.



<b>20</b>	21.1±5.7	0.05	ND	ND	23.5±3.5	0.04	30.2±6.3	0.7	33.7±2.7	0.03	18.0±5.6	0.06	1.0±0.4
<b>21</b>	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	22.2±8.4
<b>22</b>	53.1±5.2	1.2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	65.1±18.1
<b>23</b>	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	40.4±13.2
<b>24</b>	18.1±4.1	1.7	ND	ND	ND	ND	ND	ND	34.5±4.7	0.9	22.4±7.1	1.4	30.8±10.3
<b>LAP</b>	24.1±3.4	4.1	>100	1	>100	1	>100	1	>100	1	>100	1	>100 <sup>b</sup>
<b>ETP*</b>	1.30±0.7	77	24.5±8.4	4.1	2.4±0.8	42	99.3±5.2	1	>100	1	>100	1	>100 <sup>b</sup>

\*Positive control. **SI**: selectivity index,  $SI = IC_{50} \text{ PBMC} / IC_{50} \text{ tumor}$ ; <sup>a</sup>  $IC_{50}$ : concentration that reduced 50% of cell viability, values ( $\mu\text{M}$ ) mean  $\pm$  S.E.M; ND =  $IC_{50}$  values were not determined once reduction in 50% of cell viability was not reached in the tested concentrations; <sup>b</sup> Estimated SI: for compounds whose exact  $IC_{50}$  values could not be determined (<0.78 or 100) due to low solubility or even at the minimum or maximum concentration evaluated did not reach 50% reduction in cell viability, the values to estimate the SI was 0.78 or 100  $\mu\text{M}$ . LAP: lapachol. ETP: etoposide.

Lapachol (**1**) was active only against HL60 cell line with an  $IC_{50}$ = 24.1  $\mu\text{M}$  and the most of its glycosides were active against at least one tumor cell line. Classical glycosides **5** and **14-16** were active against all cancer cell lines, being more active than glycosyl triazoles **17-24** for most of the cell lines. Compound **5** was the most active against three cancer cell lines Jurkat,  $IC_{50}$ = 10.2  $\mu\text{M}$ , THP-1,  $IC_{50}$ = 11.5  $\mu\text{M}$  and MDA-MB-231,  $IC_{50}$ = 8.2  $\mu\text{M}$ . MDA-MB-231 is a cell line that lacks hormone receptors [42, 43], not responding to anti-hormonal therapy and that may acquire resistance to chemotherapy [44]. Besides, compound **5** showed the lower toxicity among all derivatives to non-tumor PBMC presenting the higher selectivity index ( $SI$ = 9.8 for HL-60) [45]. Among the classical glycosides compound **14** was active against MCF-7 (with a  $IC_{50}$ = 31.4  $\mu\text{M}$ ), an estrogen receptor (ER)-positive breast cancer cell [46, 47] and HCT-116 ( $IC_{50}$ = 23.0  $\mu\text{M}$ ), a resistant cancer cell line [48] and the second least toxic regarding classical glycosides for PBMC ( $IC_{50}$ = 5.4  $\mu\text{M}$ ). Compound **15** was the most active against HL60 with an  $IC_{50}$  value of 4.2  $\mu\text{M}$ , indicating a potent anticancer activity for this cell line. Compound **16** was the second more active against Jurkat and HCT-116, with an  $IC_{50}$  of 10.4  $\mu\text{M}$  and 24.7  $\mu\text{M}$ , respectively.

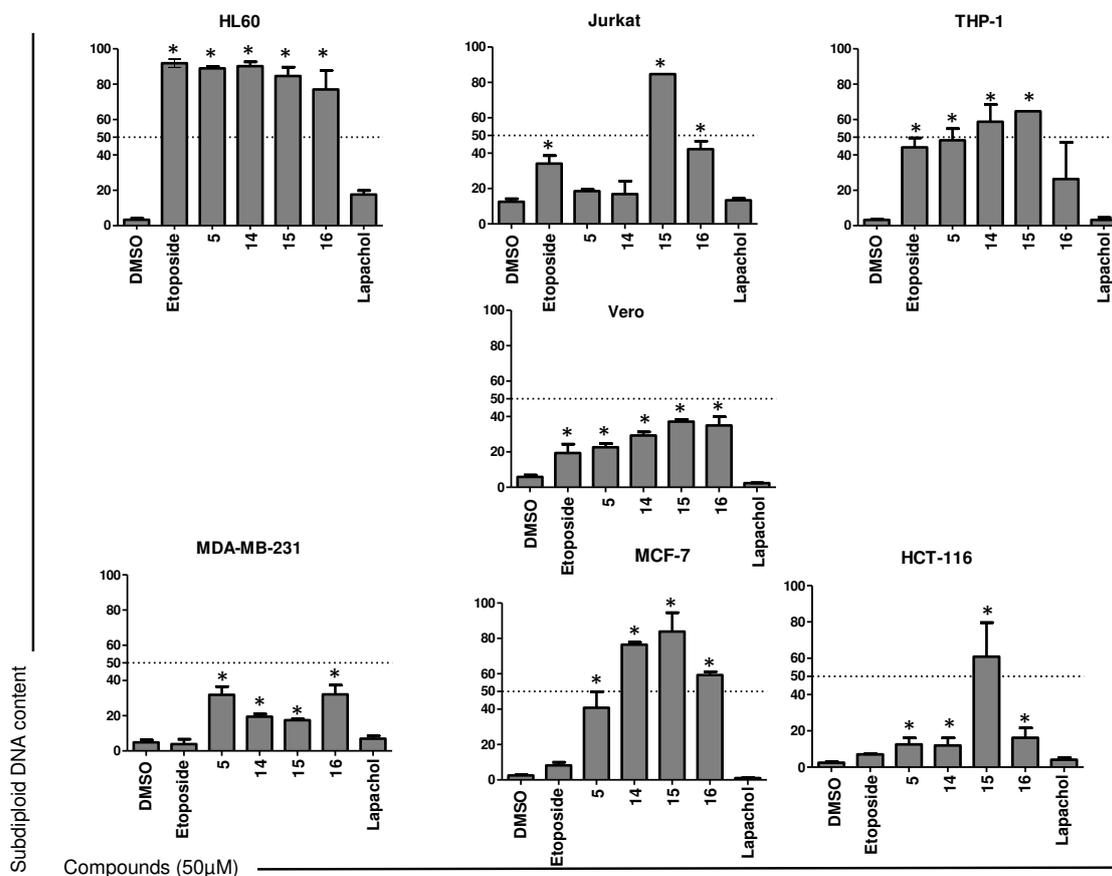
Peracetylated and deacetylated glycosyl triazoles **17-24** were active against at least two cancer cell lines. Compound **17** was active against two cancer cell lines, being the most active against both (THP-1,  $IC_{50}$ = 20.8  $\mu\text{M}$  and HL60,  $IC_{50}$ = 11.6  $\mu\text{M}$ ). Compound **18** was active against the six cancer cell lines, with higher activity against MCF-7, with an  $IC_{50}$ = 14.3  $\mu\text{M}$ . Besides, compound **18** was the only compound to show activity against Jurkat ( $IC_{50}$ = 23.0  $\mu\text{M}$ ). Compound **20** was active against all cancer cell lines, except Jurkat,

being the most active against HCT-116 ( $IC_{50}$ = 18.0  $\mu$ M), a resistant cancer cell line [48]. Compounds **18** and **20** were the only to show activity against MDA-MB-231. Among the deacetylated compounds (**21-24**), only compounds **22** and **24** (D-galactose e L-fucose derivatives, respectively) were active. Compound **22** was active against HL-60 ( $IC_{50}$ = 53.1  $\mu$ M). Compound **24** was active against HL-60 ( $IC_{50}$ = 18.1  $\mu$ M), MCF-7 ( $IC_{50}$ = 34.5  $\mu$ M) and HCT-116 ( $IC_{50}$ = 22.4  $\mu$ M). Regarding the toxicity towards PBMC cells, compounds **17**, **18**, and **20** showed cytotoxicity against PBMC at lower micromolar range (<10  $\mu$ M). Compounds **19**, **21**, **23** and **24** displayed toxicity at range of 20 to 40  $\mu$ M. Compound **22** was the less cytotoxic to PBMC cells among all new lapachol, derivatives with  $IC_{50}$ = 65  $\mu$ M.

The selectivity index (SI) is the ratio between the  $IC_{50}$  towards normal cells and cancer cells. Comparing all analogs, compound **5** was the the most selective, with SI values of 9.8 to HL60, 4.2 to Jurkat, 3.7 to THP-1, 5.2 to MDA-MB-231, 0.9 to MCF-7 and 1.7 to HCT-116. A SI greater than 3 is considered satisfactory for anticancer drugs as reported in the literature [49, 50].

#### *2.2.1.1 Evaluation of DNA fragmentation assay as indicative of cell death by apoptosis*

The subdiploid DNA quantification accomplished in this work was used as strategy to measure the DNA fragmentation, being an indicative of cell death activation by apoptosis, according described by Nicoletti et al [51, 52]. According to the protocol used, cells with DNA fragments by death apoptosis process can be evaluated by quantification of subdiploid DNA content. The classical glycosides (compounds **5** and **14-16**) presented higher cytotoxicity against the majority of tumor cell lines and the best selectivity index (SI) for at least one tumor cell lines, so they were selected for the quantification of subdiploid DNA content as indicative of the pro-apoptotic potential [51]. There was an increase in subdiploid DNA content in the tumor cell lines treated with classical lapachol glycosides **5** and **14-16**, but not with lapachol. The compounds were evaluated in the concentration of 50  $\mu$ M and glycosides induced DNA fragmentation in all tumor cell line (Fig. 7).



**Fig. 7** – Lapachol derivatives, but not Lapachol induces DNA fragmentation in tumor and non-tumor cell lines. Tumor and non-tumor cell lineages were treated with lapachol and derivatives at 50 µM for 24 h and the sub diploid DNA content was measured by flow cytometry. Representative data of three independent experiments (at least) in triplicate.

\*Statistically different of control (DMSO 0.5%),  $p < 0.05$ .

The more susceptible tumor cell line was HL60 (human myeloid leukemia) for which it was observed a high increase in subdiploid DNA content as compared with control (DMSO, 0.5%) and lapachol (1). Compound 15 showed high activity against Jurkat cells (human lymphoid leukemia), followed by compound 16. In the other cell lines lapachol did not show activity, but its derivatives were active. Compound 15 was the most active against MCF-7 (derived from ER-positive breast cancer), followed by compounds 14 and 16, inducing more than 50% of DNA fragmentation as compared to compound 5. In MDA-MB-231 cells the compounds 5 and 16 showed same percentage of sub diploid DNA content increase

(~40%), being better than compounds **14** and **15**. For HCT-116 (colorectal carcinoma) compound **15** was the only active.

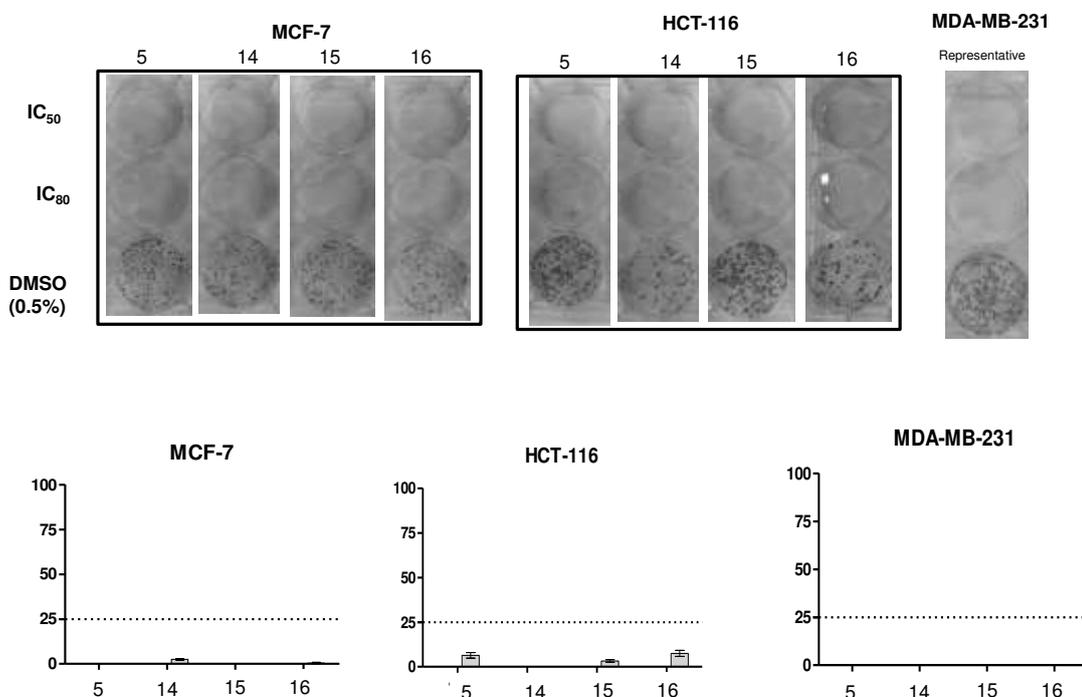
A common characteristic of cell lines used in this work is related to presence or absence of checkpoint p53 protein activity. The leukemic cell lines HL60, Jurkat and THP-1 lack p53 protein [53–55]. It is interesting to note that for HL60 and THP-1 cells the majority of compounds (except **16** for THP-1) showed similar activity. Compound **15** presented good activity against Jurkat and HCT-116 cell lines, being the most active of all compounds for these lineages.

The compounds **5** and **14** did not induce the DNA fragmentation in Jurkat (p53 null) and HCT-116 (p53 wild type) cell lines as compared to control (DMSO, 0.5%). Compound **16** increased the DNA fragmentation in all tumor cell lines (with or without p53) except HCT-116. The three cell lines from solid tumors also differ from each other in regard to p53 protein which is absent in MDA-MB-231 and present in MCF-7 and HCT-116, both p53 wild type [56–58]. When comparing the effect of compounds **14** and **15** with compounds **5** and **16**, the last were more active against MDA-MB-231 and less active against MCF-7. Compound **15** (derived from D-N-acetylglucosamine) was active against all tumor cell lines, including the lineages with protein p53 wild type (MCF-7 and HCT-116), being the only one that induced DNA fragmentation in HCT-116 as compared to lapachol and its other glycosides (**5**, **14** and **16**). The HCT-116 cell line showed p53 wild type protein [59] and the activity of chemotherapeutics may be mediated by p53 and Bax pro-apoptotic proteins which activate the apoptosis mitochondrial pathway and can activate caspase-3 [58]. On this way, the MCF-7 cells are the only ones used in this work that lack caspase-3 [60]. For MCF-7 cells other mechanisms of apoptosis induced by different chemotherapeutic agents may occur independently of caspase-3 so that DNA fragmentation can be observed despite the absence of caspase-3 [60]. Therefore, different pathways should be involved in the pro-apoptotic potential observed for new lapachol glycosides.

#### *2.2.1.2 Effect of lapachol glycosides in the clonogenic survival of solid tumor lineages*

It is well known that the DNA damage inducing agents induce cell cycle arrest at checkpoints. This is a cell survival response that allows them to repair damaged DNA and is not directly related to cell death. Cells that have the function of the p53 checkpoint protein present stop the cycle. They may show loss of viability, displaying lower values of IC<sub>50</sub>, but may show increased survival. While relative IC<sub>50</sub> values may allow

comparison between compounds, they do not predict cell survival. In addition, the assays for evaluation of subdiploid DNA content allow to evaluate cell death, but do not the cell survival after discontinuation of treatment [61]. The clonogenicity is related to the ability of a single cell to grow into a colony. Therefore, a compound that in addition to reducing cell proliferation, also inactivate clonogenic cells, shows a most efficiency. Thereby, the clonogenicity assay aims to indicate if the clonogenic tumoral cells are proliferating. And this is one characteristic responsible for tumor recurrence. To perform the clonogenicity assay, it was selected the lineages more susceptible. All compounds in the concentration of their  $IC_{50}$  values inhibited the clonogenic survival of MCF-7, HCT-116 and MDA-MB-231 after 24 h of treatment despite showing different results of DNA fragmentation in these cell lines. Compounds **5**, **14** and **16** although not inducing DNA fragmentation in HCT-116 cells, reduced their ability to form colonies, similar to compound **16**, the only one that induced DNA fragmentation in HCT-116 cells (Fig. 8). Both lapachol (**1**) and etoposide, inactive against these cells, were not evaluated in the clonogenicity assay. The activity of the new lapachol classical glycosides is similar among them, not being possible to observe differences in the colony formation in the assays, but certainly were more efficient to inhibit the survival of clonogenic cells of the three tested lineages, when compared with lapachol.



**Fig. 8** – Impact of Lapachol and derivatives on clonogenic survival of MCF-7, MDA-MB-231 and HCT-116 lineages. Cells were treated with compounds at  $IC_{50}$  and  $IC_{80}$  values for 24 h, washed, the medium was replaced and incubated for 15 days. Representative images of colony formation after treatment of MCF-7 and HCT-116 with analogs is demonstrated. To MDA-MB-231, no colonies grew after treatment with all analogs and it is demonstrated one representative image. At least, two independent experiments were

performed in triplicate. Colonies of more than 50 cells were counted, and the surviving fraction was calculated relative to control (DMSO, 0.5%) to account for basal plating efficiencies

### 2.3 Influences of the chemical structure of lapachol glycosides on bioactivity

The cytotoxicity against the evaluated cancer cell lines was in general higher for lapachol glycosides (**5**, **14-16**, **17-20**, **22** and **24**) than that of lapachol (**1**), indicating that carbohydrate moieties influenced in the cytotoxicity against tumoral and non-tumoral cells. The higher activity of peracetylated lapachol glycosides can be explained due the presence of the *O*-acetyl groups on saccharidic moiety, which impair appropriate liposolubility to the peracetylated glycosides enabling these compounds to cross the cell membrane [13]. The presence of hexose transporters on cell surface [21] may have facilitated the entry of the deacetylated lapachol glycosides that were active.

The nature of the carbohydrate moieties seems to influence on activity, wherein the variation of sugar improves or reduces the cytotoxic activity. For example, the peracetylated lapachol glycosyl triazole derived from L-fucose (**20**) was the most active against HCT-116 while its deacetylated analogue (compound **24**) was the second more active against this lineage, indicating that L-fucose residue improves the activity. The L-fucose is the only carbohydrate on the L series, all the others belonging to the D series. Unlike the other carbohydrate moieties L-fucose has a methyl group attached to C-5 of the pyranose ring, while the other compounds bear a more polar hydroxymethyl group at this position. The greater lipophilic character of the sugar portion of compound **24** apparently contributed to a better hydrophilic/lipophilic balance of the compound when compared with the other compounds of this series (**21-23**), which resulted in greater activity of **24**. The presence of triazole ring led to reduction of activity of the glycosyl triazoles as compared to classical lapachol glycosides. It is interesting to note that the presence of the acetamido (NHAc) group at C-2 in compound **15** improved its activity in regard to the other compounds as such compound showed good activity against all tumor cell lines in the DNA fragmentation assay, being the most active against Jurkat and HCT-116. For this last lineage, compound **15** was the only able to impair DNA fragmentation beyond 50 %.

### 3 Conclusions

The synthesis of 12 lapachol-based glycosides and glycosyl triazoles and evaluation against several cancer cell lines is described in the present work.

Compound **5** was far more active than lapachol (**1**) and about four to ten times more active in tumor cell lines HL60, Jurkat, THP-1 and MDA-MB-231 than in the non-tumoral PBMC cell line, which represents an important improvement in activity and selectivity as compared with lapachol (**1**). These results clearly suggest that structural modifications of the parent structure **1** with carbohydrates can produce more potent and more selective new derivatives.

Compounds **5** and **14-16** evaluated in the DNA fragmentation and clonogenicity assays showed good results in the studies of subdiploid DNA content, indicating a pro-apoptotic potential. Moreover, these compounds presented good results in the clonogenicity assays, evidencing cytotoxicity for all tumor cell lines concerning clonogenic cells, which are able of to form colonies.

The greater lipophilic character of the peracetylated classic glycosides **5**, **14-16** and glycosyl triazoles **17-20** is probably one of the main reasons for their greater activity as compared with the deacetylated ones.

In conclusion, the modification of lapachol (**1**) with glycosyl and glycosyl triazole moieties furnished compounds with greater activity and wider spectrum of action than the parent compound and can be exploited for the development of new anticancer agents.

### 4 Material and Methods

#### 4.1 Chemistry

##### 4.2.1 General procedure for the synthesis of peracetylated lapachol glycosides (**5** and **14-16**)

Lapachol (**1**, 1.0 mmol) was solubilized in 5 mL of dichloromethane and was added 5 mL of sodium carbonate 10% w/v solution and tetrabutylammonium bromide (0.3 mmol). Then a solution of the appropriate glycosyl halide (3.0 mmol) in 5 mL of dichloromethane was added. The reaction mixture was stirred at room temperature for 24h, monitoring by TLC analysis. A 6 mol L<sup>-1</sup> HCl (50 mL) solution was added until pH= 4 and the mixture was transferred to a separatory funnel. The organic layer was separated and the aqueous phase was extracted with 3 x 50 mL of dichloromethane. The combined organic layers were washed with water (3x 25 mL), dried over anhydrous sodium sulfate and concentrated under reduced

pressure. The crude classical lapachol glycosides obtained from D-glucose (**5**), D-galactose (**14**), D-N-acetylglucosamine (**15**) and L-fucose (**16**) were purified by recrystallization from methanol.

4.2.1.1 2-(2,3,4,6-tetra-O-acetyl- $\beta$ -D-glucopyranosyloxy)-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (**5**).

Yield: 60%; mp 60.2 – 66.9 °C (Lit: 62-65 °C) [13];  $[\alpha]_D^{20}$  -114.3 (*c* 0.42; CHCl<sub>3</sub>); IR  $\nu_{\max}$  2939, 1748, 1667, 1592, 1573, 1213, 1035 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  1.66 (3H, s, CH<sub>3</sub>, H-15'), 1.79 (3H, s, CH<sub>3</sub>, H-14'), 1.98–2.12 (12H, s, CH<sub>3</sub>, CH<sub>3</sub>CO), 3.29 (1H, dd, *J* = 7.0, 13.1 Hz, H-11'a), 3.37 (1H, dd, *J* = 7.7, 13.1 Hz, H-11'b), 3.76 (1H, ddd, *J* = 2.4, 4.6, 10.0 Hz, H-5), 4.06 (1H, dd, *J* = 2.2, 12.4 Hz, H-6a), 4.21 (1H, dd, *J* = 4.7, 12.4 Hz, H-6b), 5.10 (1H, t, *J* = 7.2 Hz, H-12'), 5.15 (1H, d, *J* = 9.6 Hz, H-4), 5.26 (1H, dd, *J* = 7.8 Hz, 9.4 Hz, H-2), 5.33 (1H, t, *J* = 9.3 Hz, H-3), 5.82 (1H, d, *J* = 7.7 Hz, H-1), 7.68-7.73 (2H, m, H-6' and H-7'), 8.03-8.08 (2H, m, H-5' and H-8'); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz)  $\delta$  18.0 (CH<sub>3</sub>, C-14'), 20.6-20.7 (CH<sub>3</sub>, CH<sub>3</sub>CO), 23.4 (CH<sub>2</sub>, C-11'), 25.8 (CH<sub>3</sub>, C-15'), 61.6 (CH<sub>2</sub>, C-6), 68.3 (CH, C-4), 71.7 (CH, C-2), 72.2 (CH, C-5), 72.7 (CH, C-3), 99.1 (CH, C-1), 119.3 (CH, C-12'), 126.1 (CH, C-5'), 126.5 (CH, C-8'), 131.3 (C, C-10'), 132.0 (C, C-9'), 133.5 (CH, C-6'), 134.0 (CH, C-7'), 134.2 (C, C-13'), 137.7 (C, C-3'), 152.5 (C, C-2'), 169.4-170.5 (C, COCH<sub>3</sub>), 181.0 (CO, C-4'), 184.9 (CO, C-1'); UPLC purity = 98%, tR = 7.00 min; HRMS (ESI<sup>+</sup>) *m/z* calcd for C<sub>29</sub>H<sub>33</sub>O<sub>12</sub> 573.20, found 573.25 (M+H<sup>+</sup>).

4.2.1.2 2-(2,3,4,6-tetra-O-acetyl- $\beta$ -D-galactopyranosyloxy)-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (**14**).

Yield: 60%; mp 62.5 – 65.3°C.  $[\alpha]_D^{20}$  -92.7 (*c* 0.41; CHCl<sub>3</sub>); IR  $\nu_{\max}$  2940, 1748, 1667, 1213, 1048 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  1.68 (3H, s, CH<sub>3</sub>, H-15'), 1.82 (3H, s, CH<sub>3</sub>, H-14'), 1.94–2.18 (12H, s, CH<sub>3</sub>, CH<sub>3</sub>CO), 3.32 (1H, dd, *J* = 7.2, 13.0 Hz, H-11'a), 3.39 (1H, dd, *J* = 7.7, 13.0 Hz, H-11'b), 3.95 (1H, t, *J* = 6.6 Hz, H-5), 4.05 (1H, dd, *J* = 6.4, 11.2 Hz, H-6a), 4.10 (1H, dd, *J* = 7.3, 11.3 Hz, H-6b), 5.11-5.16 (1H, m, H-12'), 5.14 (1H, dd, *J* = 3.3, 10.4 Hz, H-3), 5.42-5.46 (2H, m, H-4 and H-2), 5.74 (1H, d, *J* = 8.6 Hz, H-1), 7.68-7.74 (2H, m, H-6' and H-7'), 8.03-8.09 (2H, m, H-5' and H-8'). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz)  $\delta$  18.0 (CH<sub>3</sub>, C-14'), 20.5-20.9 (CH<sub>3</sub>, CH<sub>3</sub>C=O), 23.5 (CH<sub>2</sub>, C-11'), 25.8 (CH<sub>3</sub>, C-15'), 60.9 (CH<sub>2</sub>, C-6), 66.9 (CH, C-5), 69.2 (CH, C-4), 70.7 (CH, C-3), 71.2 (CH, C-2), 99.6 (CH, C-1), 119.4 (CH, C-12'), 126.1 (CH, C-5'), 126.5 (CH, C-8'), 131.3 (C, C-10'), 132.0 (C, C-9'), 133.4 (CH, C-6'), 134.0 (CH, C-7'), 134.0 (C,

C-13'), 137.9 (C, C-3'), 152.6 (C, C-2'), 169.7-170.2 (C,  $\underline{\text{COCH}}_3$ ), 181.0 (CO, C-4'), 184.9 (CO, C-1'); UPLC purity = 98%, tR = 6.95 min; HRMS (ESI<sup>+</sup>) *m/z* calcd for C<sub>29</sub>H<sub>33</sub>O<sub>12</sub> 573.20, found 573.25 (M+H<sup>+</sup>).

4.2.1.3 2-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy- $\beta$ -*D*-glucopyranosyloxy)-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (**15**).

Yield: 70%; mp 142.4 – 144.1 °C. [ $\alpha$ ]<sub>D</sub><sup>20</sup> -85.7° (*c* 0.42; CHCl<sub>3</sub>); IR  $\nu_{\text{max}}$  3308, 2931, 1742, 1666, 1621, 1593, 1539, 1224, 1191, 1043 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  1.66 (3H, s, CH<sub>3</sub>, H-15'), 1.79 (3H, s, CH<sub>3</sub>, H-14'), 2.02–2.07 (12H, s, CH<sub>3</sub>,  $\underline{\text{CH}}_3\text{CO}$ ), 3.31 (1H, dd, *J* = 6.8, 13.2 Hz, H-11'a), 3.43 (1H, dd, *J* = 7.9, 13.0 Hz, H-11'b), 3.64-3.68 (1H, m, H-5), 4.08 (1H, dd, *J* = 2.3, 12.3 Hz, H-6a), 4.21 (1H, dd, *J* = 4.6, 12.4 Hz, H-6b), 4.37-4.44 (1H, m, H-2), 5.11 (1H, t, *J* = 7.2 Hz, H-12'), 5.15-5.21 (2H, m, H-3 and H-4), 5.36 (1H, d, *J* = 8.6 Hz, H-1), 6.35 (1H, d, *J* = 8.6 Hz,  $\underline{\text{NHCOCH}}_3$ ), 7.70-7.76 (2H; m, H-6' and H-7'), 8.04-8.10 (2H, m, H-5' and H-8'); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz)  $\delta$  18.0 (CH<sub>3</sub>, C-14'), 20.6-20.7 (CH<sub>3</sub>,  $\underline{\text{CH}}_3\text{C=O}$ ), 23.4 (CH<sub>3</sub>,  $\underline{\text{CH}}_3\text{C=O}$  amide), 23.6 (CH<sub>2</sub>, C-11'), 25.8 (CH<sub>3</sub>, C-15'), 54.3 (CH, C-2), 61.8 (CH<sub>2</sub>, C-6), 68.1 (CH, C-5), 72.5 (CH, C-4), 73.3 (CH, C-3), 100.4 (CH, C-1), 119.2 (CH, C-12'), 126.3 (CH, C-5'), 126.7 (CH, C-8'), 131.1 (C, C-10'), 132.1 (C, C-9'), 133.6 (CH, C-6'), 134.3 (CH, C-7'), 134.4 (C, C-13'), 139.1 (C, C-3'), 153.2 (C, C-2'), 169.3 (C,  $\underline{\text{NHCOCH}}_3$ ), 170.6-171.0 (C,  $\underline{\text{COCH}}_3$ ), 181.7 (CO, C-4'), 184.8 (CO, C-1'); UPLC purity = 96%, tR = 5.93 min; HRMS (ESI<sup>+</sup>) *m/z* calcd for C<sub>29</sub>H<sub>34</sub>NO<sub>11</sub> 572.21, found 571.88 (M+H<sup>+</sup>).

4.2.1.4 2-(2,3,4-tri-*O*-acetyl-6-deoxy- $\beta$ -*L*-galactopyranosyloxy)-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (**16**).

Yield: 80%; mp 93.6 – 95.1 °C. [ $\alpha$ ]<sub>D</sub><sup>27</sup> +104.2° (*c* 0.48; CHCl<sub>3</sub>). IR  $\nu_{\text{max}}$  2961, 2858, 1748, 1668, 1618, 1594, 1216, 1195, 1056, 1021 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta$  1.14 (3H, d, *J* = 6.4 Hz, CH<sub>3</sub>, H-6), 1.68 (3H, s, CH<sub>3</sub>, H-15'), 1.83 (3H, s, CH<sub>3</sub>, H-14'), 2.02–2.20 (9H, s, CH<sub>3</sub>,  $\underline{\text{CH}}_3\text{CO}$ ), 3.33 (1H, dd, *J* = 7.0, 13.0 Hz, H-11'a), 3.43 (1H, dd, *J* = 7.8, 13.0 Hz, H-11'b), 3.83 (1H, d, *J* = 6.4 Hz, H-5), 5.11-5.18 (1H, m, H-12'), 5.12 (1H, dd, *J* = 3.4, 10.4 Hz, H-3), 5.26 (1H, d, *J* = 2.9 Hz, H-4), 5.41 (1H, dd, *J* = 7.9, 10.4 Hz, H-2), 5.70 (1H, d, *J* = 7.9 Hz, H-1), 7.68-7.73 (2H, m, H-6' and H-7'), 8.02-8.08 (2H, m, H-5' and H-8') <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz)  $\delta$  15.9 (CH<sub>3</sub>, C-6), 18.0 (CH<sub>3</sub>, C-14'), 20.6-20.9 (CH<sub>3</sub>,  $\underline{\text{CH}}_3\text{CO}$ ), 23.5 (CH<sub>2</sub>, C-11'), 25.8 (CH<sub>3</sub>, C-15'), 69.2 (CH<sub>2</sub>, C-5), 69.7 (CH, C-4), 70.1 (CH, C-3), 71.1 (CH, C-2), 99.6 (CH, C-1), 119.6 (CH, C-12'), 126.1 (CH, C-5'), 126.4 (CH, C-8'), 131.3 (C, C-10'), 132.0 (C, C-9'), 133.4 (CH, C-6'), 133.9 (C, C-7'), 133.9 (C, C-13'), 137.8 (C, C-3'), 152.9 (C, C-2'), 169.8-170.6 (C,  $\underline{\text{COCH}}_3$ ), 181.1

(CO, C-4'), 185.1 (CO, C-1'); UPLC purity = 99%, tR = 7.18 min; HRMS (ESI<sup>+</sup>) *m/z* calcd for C<sub>27</sub>H<sub>31</sub>O<sub>10</sub> 515.19, found 515.24 (M+H<sup>+</sup>).

#### 4.2.2 Synthesis of 2-[(2-propyn-1-yl)oxy]3-(3-methyl-2-butenyl)-1,4-naphthoquinone (**28**)

Lapachol (1.0 g, 4.12 mmol) was dissolved in 20 mL of CH<sub>2</sub>Cl<sub>2</sub> in a 100 mL round bottom flask, followed by addition of 20 mL of a 5% w/v K<sub>2</sub>CO<sub>3</sub> solution (1.0 g, 7.24 mmol) and the mixture was stirred at room temperature for 30 minutes. Tetra-*n*-butylammonium bromide (0.1 g, 0.31 mmol) and propargyl bromide (1.0 mL, 6.72 mmol) were added and the reaction mixture was stirred at room temperature. After six hours additional propargyl bromide (0.50 mL, 3.36 mmol) was added and evolution of the reaction was monitored by TLC analysis. After 24 h, the reaction mixture was transferred to a separatory funnel, the organic phase was separated and the aqueous phase was extracted with 3 x 50 mL CH<sub>2</sub>Cl<sub>2</sub>. The combined organic phases were washed with water (6 x 50 mL), dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated to dryness. The crude product was treated with hexane (200 mL) and the solvent was evaporated to give a solid that was recrystallized from hot methanol to give compound **28** as a yellow solid.

Yield: 67%; mp 39.4 – 41.4 °C (Lit: 54-55 °C) [62]; IR  $\nu_{\max}$  3355, 3279, 2964, 2853, 2124, 1660, 1640, 1610, 1593, 1579, 1334, 1186, 1047 cm<sup>-1</sup>, <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  1.68 (3H, s, CH<sub>3</sub>, H-15), 1.80 (3H, s, CH<sub>3</sub>, H-14), 2.51 (1H, t, *J* = 2.4 Hz, H-18), 3.36 (2H, d, *J* = 7.2 Hz, H-11), 5.13 (2H, d, *J* = 2.4 Hz, H-16), 5.15-5.17 (1H, m, H-12), 7.67-7.72 (2H, m, H-6 and H-7), 8.04-8.09 (2H, m, H-5 and H-8); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz)  $\delta$  18.0 (CH<sub>3</sub>, C-14), 23.3 (CH<sub>2</sub>, C-11), 25.8 (CH<sub>2</sub>, C-15), 60.3 (CH<sub>2</sub>, C-16), 76.4 (C, C-17), 78.4 (CH, C-18), 119.8 (CH, C-12), 126.2 (CH, C-5), 126.4 (CH, C-8), 131.4 (C, C-9), 132.1 (C, C-9), 133.3 (CH, C-6), 133.9 (CH, C-7), 133.9 (C, C-13), 136.8 (CH, C-3), 155.0 (C, C-2), 181.7 (CO, C-1), 185.1 (CO, C-4).

#### 4.2.3 General procedure for the synthesis of lapachol glycosyl triazoles (**17-24**)

To a 50 mL round bottom flask was added **28** (0.30 mmol) dissolved in 1 mL of tetrahydrofuran, followed by the appropriate glycosyl azide (0.27 mmol), dissolved in 0.5 mL of tetrahydrofuran. Then, Cu(OAc)<sub>2</sub>·H<sub>2</sub>O 50% mol, dissolved in 0.5 mL of water and sodium ascorbate 60% mol, dissolved in 1 mL of water were added in a stepwise manner. The reaction mixture was stirred at room temperature for 4 h and monitored by TLC analysis. The tetrahydrofuran was removed by distillation at reduced pressure. For

peracetylated glycosyl triazoles the reaction residues were solubilized in 50 mL CH<sub>2</sub>Cl<sub>2</sub> and washed with 2 x 50 mL H<sub>2</sub>O and subsequently washed with 3 x 50 mL alkaline EDTA 20% w/v. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub> and filtered. The organic phase was removed by distillation at reduced pressure. The deacetylated glycosyl triazoles (**21-24**) were purified directly. The derivatives **17-20** were added to Florisil and purified with silica column with following mobile phase (CH<sub>2</sub>Cl<sub>2</sub>: ethyl acetate/4:6) and the deacetylated using ethyl acetate: MeOH/9:1 as mobile phase.

4.2.3.1 2-[1-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-1,2,3-triazol-4-(methyl)oxy]-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (**17**).

Yield: 68%; mp 158.5 – 159.6 °C.  $[\alpha]_D^{20}$  -39.4 (c 0.38; acetone); IR  $\nu_{\max}$  2914, 1739, 1667, 1652, 1605, 1254, 1195, 1040 cm<sup>-1</sup>; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz):  $\delta$  1.62 (3H, s, CH<sub>3</sub>, H-15'), 1.72 (3H, s, CH<sub>3</sub>, H-14'), 1.98–2.05 (12H, s, CH<sub>3</sub>, CH<sub>3</sub>CO), 3.05 (2H, d, *J* = 8.0 Hz, H-11'), 4.08 (1H, dd, *J* = 2.4, 12.4 Hz, H-6a), 4.14 (1H, dd, *J* = 5.2, 12.4 Hz, H-6b), 4.36–4.40 (1H, m, H-5), 4.94 (1H, t, *J* = 7.2 Hz, H-12'), 5.19 (1H, d, *J* = 9.6 Hz, H-4), 5.49–5.59 (3H, m, H-2 and H-16'), 5.65 (1H, t, *J* = 9.2 Hz, H-3), 6.37 (1H, d, *J* = 9.2 Hz, H-1), 7.85–7.89 (2H, m, H-6' and H-7'), 8.00 (1H, dd, *J* = 4.0, 8.0 Hz, H-5'), 8.04 (1H, dd, *J* = 4.0, 8.0 Hz, H-8'), 8.55 (1H, s, H-18'); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 100 MHz)  $\delta$  18.1 (CH<sub>3</sub>, C-14'), 20.1–20.9 (CH<sub>3</sub>, CH<sub>3</sub>CO), 23.1 (CH<sub>2</sub>, C-11'), 25.9 (CH<sub>3</sub>, C-15'), 62.3 (CH<sub>2</sub>, C-6), 65.5 (CH<sub>2</sub>, C-16'), 68.0 (CH, C-4), 70.6 (CH, C-2), 72.5 (CH, C-5), 73.7 (CH, C-3), 84.3 (CH, C-1), 120.4 (CH, C-12'), 124.4 (CH, C-18'), 126.2 (CH, C-5'), 126.4 (CH, C-8'), 131.6 (C, C-10'), 131.8 (C, C-9'), 133.1 (CH, C-17'), 134.2 (CH, C-6'), 134.6 (CH, C-7'), 135.0 (C, C-13'), 143.7 (C, C-3'), 156.2 (C, C-2'), 168.8–170.4 (C, COCH<sub>3</sub>), 181.6 (CO, C-1'), 185.0 (CO, C-4'); UPLC purity = 95%, tR = 6.84 min; HRMS (ESI<sup>+</sup>) *m/z* calcd for C<sub>32</sub>H<sub>36</sub>N<sub>3</sub>O<sub>12</sub> 654.23, found 655.18 (M+H<sup>+</sup>).

4.2.3.2 2-[1-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-1,2,3-triazol-4-(methyl)oxy]-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (**18**).

Yield: 57%; mp 128.4–130.6 °C.  $[\alpha]_D^{20}$  -4.8 (c 0.42; CH<sub>2</sub>Cl<sub>2</sub>); IR  $\nu_{\max}$  3078, 2967, 1743, 1664, 1646, 1596, 1221, 1043 cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  1.66 (3H, s, CH<sub>3</sub>, H-15'), 1.72 (3H, s, CH<sub>3</sub>, H-14'), 1.79–2.22 (12H, s, CH<sub>3</sub>, CH<sub>3</sub>CO), 3.25 (2H, d, *J* = 7.2 Hz, H-11'), 4.10–4.24 (3H, m, H-5 and H-6), 5.06 (1H, t, *J* = 7.1 Hz, H-12'), 5.24 (1H, dd, *J* = 3.2, 10.3 Hz, H-3), 5.53 (1H, t, *J* = 9.5 Hz, H-2), 5.55 (1H, d, *J* = 3.4 Hz, H-4), 5.58 (2H, br, H-16'), 5.83 (1H, d, *J* = 9.3 Hz, H-1), 7.69–7.71 (2H, m, H-6' and H-7'), 7.98

(1H, s, H-18'), 8.06 (1H, d,  $J = 4.8$  Hz, H-5'), 8.08 (1H, d,  $J = 4.8$  Hz, H-8');  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz)  $\delta$  17.9 ( $\text{CH}_3$ , C-14'), 20.0-20.6 ( $\text{CH}_3$ ,  $\underline{\text{CH}_3\text{CO}}$ ), 23.2 ( $\text{CH}_2$ , C-11'), 25.8 ( $\text{CH}_3$ , C-15'), 61.2 ( $\text{CH}_2$ , C-6), 65.8 ( $\text{CH}_2$ , C-16'), 66.9 (CH, C-5), 67.8 (CH, C-4), 70.7 (CH, C-3), 74.2 (CH, C-2), 86.4 (CH, C-1), 118.9 (CH, C-12'), 122.0 (CH, C-18'), 126.2 (CH, C-5'), 126.3 (CH, C-8'), 131.5 (C, C-10'), 132.1 (C, C-9'), 133.2 (CH, C-6'), 133.7 (C, C-13'), 133.8 (CH, C-7'), 135.9 (C, C-3'), 155.7 (C, C-2'), 168.9-170.3 (C,  $\underline{\text{COCH}_3}$ ), 182.0 (CO, C-1'), 185.2 (CO, C-4'); UPLC purity >99%, tR = 6.79 min; HRMS (ESI<sup>+</sup>)  $m/z$  calcd for  $\text{C}_{32}\text{H}_{36}\text{N}_3\text{O}_{12}$  654.23, found 653.87 (M+H<sup>+</sup>).

4.2.3.3 2-[1-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy- $\beta$ -D-glucopyranosyl)-1,2,3-triazol-4-(methyl)oxy]-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (**19**).

Yield: 68%; mp 218.5 – 222.1 °C.  $[\alpha]_{\text{D}}^{20}$  -27.5 ( $c$  0.40;  $\text{CH}_2\text{Cl}_2$ ); IR  $\nu_{\text{max}}$  3291, 2936, 1749, 1661, 1596, 1534, 1213, 1190, 1039  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR ( $\text{DMSO-}d_6$ , 400 MHz)  $\delta$  1.52 (3H, s,  $\text{CH}_3$ , H-15'), 1.58 (3H, s,  $\text{CH}_3$ , H-14'), 1.60 (3H, s,  $\text{CH}_3$ ,  $\underline{\text{NHCOCH}_3}$ ), 1.94–2.01 (9H, s,  $\text{CH}_3$ ,  $\underline{\text{CH}_3\text{CO}}$ ), 3.03 (2H, d,  $J = 6.8$  Hz, H-11'), 4.05 (1H, d,  $J = 11.8$  Hz, H-6a), 4.14 (1H, dd,  $J = 4.9, 12.3$  Hz, H-6b), 4.24 (1H, dd,  $J = 2.8, 9.8$  Hz, H-5), 4.61 (1H, q,  $J = 9.7$  Hz, H-2), 4.95 (1H, t,  $J = 6.6$  Hz, H-12'), 5.09 (1H, t,  $J = 9.7$  Hz, H-3), 5.33 (1H, t,  $J = 9.8$  Hz, H-4), 5.47 (1H, d,  $J = 13.0$  Hz, H-16'a), 5.51 (1H, d,  $J = 12.9$  Hz, H-16'b), 6.10 (1H, d,  $J = 9.9$  Hz, H-1), 7.83-7.85 (2H, m, H-6' and H-7'), 7.96-7.98 (1H, m,  $\underline{\text{NHCOCH}_3}$ ), 8.01-8.04 (2H, m, H-5' and H-8'), 8.41 (1H, s, H-18').  $^{13}\text{C}$  NMR ( $\text{DMSO-}d_6$ , 100 MHz)  $\delta$  17.6 ( $\text{CH}_3$ , C-14'), 20.2-22.0 ( $\text{CH}_3$ ,  $\underline{\text{CH}_3\text{CO}}$ ), 22.6 ( $\text{CH}_2$ , C-11'), 25.3 ( $\text{CH}_3$ , C-15'), 52.0 ( $\text{CH}_2$ , C-2), 61.8 ( $\text{CH}_2$ , C-6), 65.1 ( $\text{CH}_2$ , C-16'), 68.0 (CH, C-5), 72.4 (CH, C-4), 73.3 (CH, C-3), 84.8 (CH, C-1), 119.9 (CH, C-12'), 123.6 (CH, C-18'), 125.7 (CH, C-5'), 125.9 (CH, C-8'), 131.1 (C, C-10'), 131.4 (C, C-9'), 132.5 (CH, C-17'), 133.7 (CH, C-6'), 134.1 (CH, C-7'), 134.4 (C, C-13'), 142.8 (C, C-3'), 155.8 (C, C-2'), 169.2-169.9 (C,  $\underline{\text{CH}_3\text{CO}}$ ), 181.1 (CO, C-1'), 184.5 (CO, C-4'); UPLC purity = 95%, tR = 6.10 min; HRMS (ESI<sup>+</sup>)  $m/z$  calcd for  $\text{C}_{32}\text{H}_{37}\text{N}_4\text{O}_{11}$  653.25, found 653.02 (M+H<sup>+</sup>).

4.2.3.4 2-[1-(2,3,4-tri-O-acetyl-6-deoxy- $\beta$ -L-galactopyranosyl)-1,2,3-triazol-4-(methyl)oxy]-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (**20**).

Yield: 85%; mp 120.3 – 124.3 °C.  $[\alpha]_{\text{D}}^{20}$  +2.5 ( $c$  0.40; acetone). IR  $\nu_{\text{max}}$  3082, 2936, 1745, 1665, 1612, 1596, 1251, 1191, 1045, 1020  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)  $\delta$  1.26 (3H, d,  $J = 8.0$  Hz,  $\text{CH}_3$ , H-6), 1.67 (3H, s,  $\text{CH}_3$ , H-15'), 1.72 (3H, s,  $\text{CH}_3$ , H-14'), 1.79–2.24 (9H, s,  $\text{CH}_3$ ,  $\underline{\text{CH}_3\text{CO}}$ ), 3.24 (2H, d,  $J = 4.0$  Hz, H-

11'), 4.12 (1H, q,  $J = 8.0$  Hz, H-5), 5.10 (1H, t,  $J = 7.2$  Hz, H-12'), 5.28 (1H, dd,  $J = 3.2, 10.2$  Hz, H-3), 5.44 (1H, t,  $J = 3.2$  Hz, H-4), 5.54 (1H, q,  $J = 9.6$  Hz, H-2), 5.63 (1H, br, H-16'), 5.85 (1H, d,  $J = 9.2$  Hz, H-1), 7.68-7.72 (2H, m, H-6' and H-7'), 8.00 (1H, s, H-18'), 8.05-8.09 (2H, m, H-5' and H-8');  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz)  $\delta$  16.0 ( $\text{CH}_3$ , C-6), 17.9 ( $\text{CH}_3$ , C-14'), 20.1-20.7 ( $\text{CH}_3$ ,  $\underline{\text{C}}\text{H}_3\text{CO}$ ), 23.2 ( $\text{CH}_2$ , C-11'), 25.8 ( $\text{CH}_3$ , C-15'), 65.8 ( $\text{CH}_2$ , C-16'), 67.9 ( $\text{CH}$ , C-5), 69.9 ( $\text{CH}_2$ , C-2), 71.2 ( $\text{CH}$ , C-4), 72.8 ( $\text{CH}$ , C-3), 86.5 ( $\text{CH}$ , C-1), 119.9 ( $\text{CH}$ , C-12'), 126.2 ( $\text{CH}$ , C-5' and C-8'), 131.5 (C, C-10'), 132.1 (C, C-9'), 133.2 ( $\text{CH}$ , C-6'), 133.7 ( $\text{CH}$ , C-7'), 133.8 (C, C-13'), 135.8 (C, C-3'), 155.8 (C, C-2'), 169.0-170.3 (C,  $\text{CH}_3\text{C}\underline{\text{O}}$ ), 181.9 (CO, C-1'), 184.2 (CO, C-4'); UPLC purity = 96%, tR = 6.89 min; HRMS (ESI<sup>+</sup>)  $m/z$  calcd for  $\text{C}_{30}\text{H}_{34}\text{N}_3\text{O}_{10}$  596.22, found 595.79 (M+H<sup>+</sup>).

4.2.3.5 2-[1-( $\beta$ -D-glucopyranosyl)-1,2,3-triazol-4-(methyl)oxy]-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (**21**).

Yield: 59%; mp 118.5 – 120.7 °C.  $[\alpha]_{\text{D}}^{20}$  -15.8 ( $c$  0.38; MeOH); IR  $\nu_{\text{max}}$  3282, 2912, 1652, 1593, 1092, 1047  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR ( $\text{DMSO-}d_6$ , 400 MHz)  $\delta$  1.60 (3H, s,  $\text{CH}_3$ , H-15'), 1.64 (3H, s,  $\text{CH}_3$ , H-14'), 3.11 (2H, d,  $J = 7.2$  Hz, H-11'), 3.21-3.47 (4H, m, H-3, H-4 and H-6), 3.68-3.78 (2H, m, H-2 and H-5), 4.58 (1H, t,  $J = 5.2$  Hz, OH), 4.99 (1H, t,  $J = 7.2$  Hz, H-12'), 5.13 (1H, d,  $J = 5.6$  Hz, OH), 5.25 (1H, d,  $J = 4.8$  Hz, OH), 5.34 (1H, d,  $J = 6.4$  Hz, OH), 5.46 (2H, s, H-16'), 5.55 (1H, d,  $J = 9.2$  Hz, H-1), 7.83-7.87 (2H, m, H-6' and H-7'), 7.96-8.04 (2H, m, H-5' and H-8'), 8.42 (1H, s, H-18');  $^{13}\text{C}$  NMR ( $\text{DMSO-}d_6$ , 100 MHz)  $\delta$  17.7 ( $\text{CH}_3$ , C-14'), 22.6 ( $\text{CH}_2$ , C-11'), 25.4 ( $\text{CH}_3$ , C-15'), 60.6 ( $\text{CH}_2$ , C-6), 65.5 ( $\text{CH}_2$ , C-16'), 69.5 ( $\text{CH}$ , C-4), 72.0 ( $\text{CH}$ , C-2), 76.9 ( $\text{CH}$ , C-5), 79.9 ( $\text{CH}$ , C-3), 87.4 ( $\text{CH}$ , C-1), 120.0 ( $\text{CH}$ , C-12'), 123.9 ( $\text{CH}$ , C-18'), 125.6 ( $\text{CH}$ , C-5'), 125.9 ( $\text{CH}$ , C-8'), 131.1 (C, C-10'), 131.3 (C, C-9'), 132.6 ( $\text{CH}$ , C-17'), 133.7 ( $\text{CH}$ , C-6'), 134.1 ( $\text{CH}$ , C-7'), 134.1 (C, C-13'), 142.5 (C, C-3'), 156.2 (C, C-2'), 181.0 (CO, C-1'), 184.6 (CO, C-4'); UPLC purity >99%, tR = 4.53 min; HRMS (ESI<sup>+</sup>)  $m/z$  calcd for  $\text{C}_{24}\text{H}_{28}\text{N}_3\text{O}_8$  486.19, found 485.84 (M+H<sup>+</sup>).

4.2.3.6 2-[1-( $\beta$ -D-galactopyranosyl)-1,2,3-triazol-4-(methyl)oxy]-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (**22**).

Yield: 51%; mp 182.5 - 187.2 °C.  $[\alpha]_{\text{D}}^{20}$  -8.0 ( $c$  1.00; MeOH); IR  $\nu_{\text{max}}$  3282, 2912, 1652, 1593, 1092, 1047  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR ( $\text{DMSO-}d_6/\text{acetone-}d_6$ , 400 MHz)  $\delta$  1.63 (3H, s,  $\text{CH}_3$ , H-15'), 1.69 (3H, s,  $\text{CH}_3$ , H-14'), 3.19 (2H, d,  $J = 7.4$  Hz, H-11'), 3.59-3.70 (3H, m, H-3 and H-6), 3.81 (1H, t,  $J = 6.0$  Hz, H-5), 3.95 (1H, t,  $J =$

3.1 Hz, H-4), 4.16-4.22 (1H, m, H-2), 4.48-4.51 (1H, m, OH), 4.79 (1H, d,  $J = 5.8$  Hz, OH), 5.04 (1H, t,  $J = 6.9$  Hz, H-12'), 5.08 (1H, d,  $J = 5.7$  Hz, OH), 5.56 (2H, s, H-16'), 5.59 (1H, d,  $J = 9.2$  Hz, H-1), 7.84-7.86 (2H, m, H-6' and H-7'), 8.02-8.10 (2H, m, H-5' and H-8'), 8.39 (1H, s, H-18');  $^{13}\text{C}$  NMR (DMSO- $d_6$ /acetone- $d_6$ , 100 MHz)  $\delta$  18.1 (CH<sub>3</sub>, C-14'), 23.5 (CH<sub>2</sub>, C-11'), 25.8 (CH<sub>3</sub>, C-15'), 61.4 (CH<sub>2</sub>, C-6), 66.6 (CH<sub>2</sub>, C-16'), 69.4 (CH, C-5), 70.8 (CH, C-4), 75.1 (CH, C-3), 79.4 (CH, C-2), 89.3 (CH, C-1), 120.9 (CH, C-12'), 124.1 (CH, C-18'), 126.5 (CH, C-5'), 126.7 (CH, C-8'), 132.2 (C, C-10'), 132.6 (C, C-9'), 133.6 (CH, C-17'), 134.3 (CH, C-6'), 134.7 (CH, C-7'), 134.7 (C, C-13'), 135.6 (C, C-3'), 157.2 (C, C-2'), 182.2 (CO, C-1'), 185.5 (CO, C-4'); UPLC purity >99%, tR = 4.47 min; HRMS (ESI<sup>+</sup>)  $m/z$  calcd for C<sub>24</sub>H<sub>28</sub>N<sub>3</sub>O<sub>8</sub> 486.19, found 485.84 (M+H<sup>+</sup>).

4.2.3.7 2-[1-(2-acetamido-2-deoxy- $\beta$ -D-glucopyranosyl)-1,2,3-triazol-4-(methyl)oxy]-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (**23**).

Yield: 55%; mp 174.9 – 178.5 °C.  $[\alpha]_{\text{D}}^{20}$  -16.0 (c 0.50; MeOH); IR  $\nu_{\text{max}}$  3485, 3282, 2919, 1655, 1593, 1533, 1085, 1049 cm<sup>-1</sup>;  $^1\text{H}$  NMR (DMSO- $d_6$ /acetone- $d_6$ , 400 MHz)  $\delta$  1.59 (3H, s, CH<sub>3</sub>, H-15'), 1.63 (3H, s, CH<sub>3</sub>, H-14'), 1.70 (3H, s, CH<sub>3</sub>, NHCOCH<sub>3</sub>), 3.45-3.82 (7H, m, H-3, H-4, H-5, H-6 and H-11'), 4.20 (1H, q,  $J = 5.6$  Hz, H-2), 4.33 (1H, t,  $J = 5.7$  Hz, OH), 5.04-5.05 (3H, m, H-12' and OH), 5.56 (1H, d,  $J = 12.4$  Hz, H-16'a), 5.60 (1H, d,  $J = 12.3$  Hz, H-16'b), 5.80 (1H, d,  $J = 10.0$  Hz, H-1), 7.68 (1H, d,  $J = 9.2$  Hz, NHCOCH<sub>3</sub>), 7.82-7.85 (2H, m, H-6' and H-7'), 8.01-8.09 (2H, m, H-5' and H-8'), 8.25 (1H, s, H-18');  $^{13}\text{C}$  NMR (DMSO- $d_6$ /acetone- $d_6$ , 100 MHz)  $\delta$  18.1 (CH<sub>3</sub>, C-14'), 22.8 (CH<sub>3</sub>, NHCOCH<sub>3</sub>), 23.6 (CH<sub>2</sub>, C-11'), 25.9 (CH<sub>3</sub>, C-15'), 55.5 (CH, C-2), 62.0 (CH<sub>2</sub>, C-6), 66.2 (CH<sub>2</sub>, C-16'), 71.2 (CH, C-5), 75.2 (CH, C-4), 81.0 (CH, C-3), 87.5 (CH, C-1), 121.0 (CH, C-12'), 124.0 (CH, C-18'), 126.5 (CH, C-5'), 126.7 (CH, C-8'), 132.4 (C, C-10'), 132.7 (C, C-9'), 133.5 (CH, C-17'), 134.2 (CH, C-6'), 134.6 (CH, C-7'), 134.6 (C, C-13'), 135.8 (C, C-3'), 156.8 (C, C-2'), 170.1 (C, NHCOCH<sub>3</sub>), 182.3 (CO, C-1'), 185.5 (CO, C-4'); UPLC purity >99%, tR = 4.52 min; HRMS (ESI<sup>+</sup>)  $m/z$  calcd for C<sub>26</sub>H<sub>31</sub>N<sub>4</sub>O<sub>8</sub> 527.21, found 527.00 (M+H<sup>+</sup>).

4.2.3.8 2-[1-(6-deoxy- $\beta$ -L-galactopyranosyl)-1,2,3-triazol-4-(methyl)oxy]-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (**24**).

Yield: 47%; mp 91.0-94.0 °C.  $[\alpha]_{\text{D}}^{20}$  +15.8 (c 0.38; acetone); IR  $\nu_{\text{max}}$  3357, 2926, 1666, 1090 cm<sup>-1</sup>;  $^1\text{H}$  NMR (acetone- $d_6$ , 400 MHz)  $\delta$  1.26 (3H, d,  $J = 4.0$  Hz, CH<sub>3</sub>, H-6), 1.62 (3H, s, CH<sub>3</sub>, H-15'), 1.68 (3H, s, CH<sub>3</sub>, H-14'), 3.20 (2H, d,  $J = 8.0$  Hz, H-11'), 3.76-3.79 (2H, m, H-3 and H-4), 3.95-4.03 (1H, m, H-2), 4.01 (1H,

q,  $J = 4.0$  Hz, H-5), 4.19-4.23 (2H, m, OH), 4.46 (1H, d,  $J = 4.0$  Hz, OH), 5.03 (1H, d,  $J = 8.0$  Hz, H-12'), 5.57 (2H, s, H-16'), 5.58 (1H, d,  $J = 8.0$  Hz, H-1), 7.81-7.84 (2H, m, H-6' and H-7'), 8.01-8.04 (2H, m, H-5' and H-8'), 8.25 (1H, s, H-18');  $^{13}\text{C}$  NMR (acetone- $d_6$ , 100 MHz): 15.9 (CH<sub>3</sub>, C-6), 17.2 (CH<sub>3</sub>, C-14'), 22.8 (CH<sub>2</sub>, C-11'), 24.9 (CH<sub>3</sub>, C-15'), 65.8 (CH<sub>2</sub>, C-16'), 70.1 (CH, C-5), 71.5 (CH, C-2), 73.6 (CH, C-4), 74.5 (CH, C-3), 88.4 (CH, C-1), 120.2 (CH, C-12'), 122.8 (CH, C-18'), 125.8 (CH, C-5'), 125.9 (CH, C-8'), 131.7 (C, C-10'), 132.0 (C, C-9'), 132.8 (CH, C-17'), 133.4 (CH, C-6'), 133.9 (CH, C-7'), 133.9 (C, C-13'), 135.0 (C, C-3'), 156.4 (C, C-2'), 181.5 (CO, C-1'), 184.7 (CO, C-4'); UPLC purity > 99%, tR = 5.01 min; HRMS (ESI<sup>+</sup>)  $m/z$  calcd for C<sub>24</sub>H<sub>28</sub>N<sub>3</sub>O<sub>7</sub> 470.19, found: 470.32 (M+H<sup>+</sup>).

#### 4.4 UPLC/MS analysis of lapachol derivatives

All final compounds were purified to > 95% purity, as determined by UPLC/MS analyses, carried out using an ACQUITY Ultra Performance LC<sup>TM</sup> system (Waters, Milford, MA, USA) linked simultaneously to both a PDA 2996 photodiode array detector (Waters, Milford, MA, USA) and an ACQUITY TQ Detector (Waters MS Technologies, Manchester, UK), equipped with a Z-spray electrospray ionization (ESI) source operating in positive mode. MassLynx<sup>TM</sup> software (version 4.1, Waters, Milford, MA, USA) was used to control the instruments, as well as for data acquisition and processing. Sample solutions (3  $\mu\text{L}$ ; 0.5 mg/mL) were injected into a reversed phase column (BEH<sub>C18</sub>, 1.7  $\mu\text{m}$ , 1 $\times$ 50 mm, Waters, Milford, MA), which was maintained at 40°C. The mobile phase consisted of solvent A (H<sub>2</sub>O/0.1 HCOOH) and solvent B (acetonitrile/0.1 HCOOH) at a flow rate of 300  $\mu\text{L}/\text{min}$ : T=0 min, 5% B; T=10 min, 95% B; T=11 min, 5% B; T=13 min, 5% B. The effluent was introduced into a PDA detector (scanning range 210–400 nm, resolution 1.2 nm) and subsequently into an electrospray source (source block temperature 120°C, desolvation temperature 350°C, capillary voltage 3.5 kV, cone voltage 30 V) and nitrogen was used as the desolvation gas (600 L/h). Mass chromatograms were recorded in the positive and negative ionization mode in the range from 100-1300 Da.

#### 4.5 Infrared spectroscopy

IR spectrum was recorded on a Spectrum One, Perkin-Elmer ATR system.

#### 4.6 NMR analysis

$^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, DEPT-135,  $^1\text{H}$ - $^1\text{H}$  COSY, HSQC and HMBC spectra were recorded on Bruker Avance DRX-400 ( $^1\text{H}$  400MHz and  $^{13}\text{C}$  100 MHz) in acetone- $d_6$ ,  $\text{CDCl}_3$  and  $\text{DMSO-}d_6$  at 300K using TMS as internal standard for both nuclei. Chemical shifts ( $\delta$ ) are given in ppm and  $J$  couplings in Hertz (Hz).

#### 4.7 Cytotoxicity measurements

##### 4.7.1. Cell lines and culture

Human acute promyelocytic leukemia cells (HL60), human acute T cells leukemia (Jurkat) and human monocytic leukemia cells (THP-1) were kindly donated by Prof. Gustavo P Amarante Mendes (University of São Paulo/Brazil). Human breast cancer (MCF-7 and MDA-MB-231) and colorectal lineages (HCT-116) were gently donated by Prof. Marcel Leist (University of Konstanz/Germany). Leukemia cells were cultivated in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO), supplemented with 100 U/mL penicillin and 100  $\mu\text{g}/\text{mL}$  streptomycin (GIBCO BRL, Grand Island, NY), enriched with 2 mM of L-glutamine (GIBCO UK, Grand Island, NY) and 10% fetal bovine serum. Breast cancer and colorectal lineages were cultivated in DMEM medium (Sigma-Aldrich, St. Louis, MO), supplemented with 100 U/mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin (GIBCO BRL, Grand Island, NY), enriched with 10% fetal bovine serum (GIBCO BRL, Grand Island, NY). All cultures were maintained at 37 °C in a humidified incubator with 5%  $\text{CO}_2$ . Cells were split twice a week and routinely evaluated for contamination.

##### 4.7.2. Cytotoxic of lapachol and glycosides against HL60, Jurkat, THP-1, MDA-MB-231, MCF-7 and HCT-116 cells

Cell viability was detected by the rate of the mitochondrial reduction of the yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich, St. Louis, MO) into insoluble purple formazan crystals, and the color intensity of the formazan dye is correlated with the number of viable cells [38]. Briefly, leukemia cells were seeded at density of  $5 \times 10^4$  (HL60 and THP-1) and  $1 \times 10^5$  (Jurkat). MCF-7, MDA-MB-231 and HCT-116 cells were seeded at  $1 \times 10^4$  cells/well, in 96-well plates. The plates were pre-incubated in a 95% air-humidified atmosphere with a 5%  $\text{CO}_2$  for 24 h at 37°C to allow for the adaptation of cells. The compounds were tested over a two-fold serial dilution concentration (100-

0.78 $\mu$ M). The cells cultures were incubated for an additional 48 h at 37°C with the compounds. DMSO at 0.5% was used as negative control (solvent control). After incubation, 20  $\mu$ L MTT solution (2.5 mg/mL) were added in each well followed by 4 h incubation. The supernatant was removed, and 200  $\mu$ L of 0.04 M HCl in isopropyl alcohol was added. The optical densities (OD) were measured at 595 nm in a plate reader (VarioScan, Thermo Scientific®). The results were normalized by solvent control (DMSO at 0.5%), and half maximal inhibitory concentration (IC<sub>50</sub>) and IC<sub>80</sub> values were obtained from concentration–effect curves using Prism 7.0 (GraphPad Software Inc). Etoposide was used as positive control. Three experiments were performed in triplicate.

#### *4.7.3. Cytotoxic of lapachol and derivates against human peripheral blood mononuclear cells*

##### *4.7.3.1. Human peripheral blood mononuclear cells*

Cells obtained from six healthy donors were collected and processed in less than 24h intervals. Protocol for the use of human peripheral blood at COEP number 30860113.1.1001.5149/2016.

##### *4.7.3.2. Isolation of human peripheral blood mononuclear cells*

Human peripheral blood mononuclear cells (PBMC) were separated according to the method described by Souza-Fagundes et al (2002), with modifications [63]. Cells were isolated from venous blood collected from six healthy subjects using heparinized tubes. The heparinized blood was gently placed in 50mL Falcon tubes (Pyrex Laboratory Glassware) containing a mixture of Ficoll-diatrizoate (LSM-Lymphocyte Separation Medium, Organon Teknika Corporation, Durhan) in the ratio of a part of Ficoll-diatrizoate to two parts of blood. This preparation was then centrifuged for 40 minutes, 500 g at 20°C. After centrifugation, the cells were removed and transferred to a 15 mL graduated conical tube. Then the cells were washed with RPMI for 7 minutes, 300 g at 4°C, three times. Cells were seed at density of 2x10<sup>5</sup> cells/well (96-well plates). Cells were maintained in complete culture medium containing RPMI (GIBCO, UK), supplemented with 10% v/v fetal bovine serum, previously inactivated (Gibco, Brazil), 2mM L-Glutamine (1% v/v) (200 mM stock solution, GIBCO UK, Grand Island, NY), 1% antibiotic-antimycotic mixture (stock solution 1000 U/mL penicillin, 1000  $\mu$ g/mL streptomycin and 25 $\mu$ g/mL fungizone). After stabilization, all cells were incubated with the substances at two-fold serial dilution concentration (100-0.78 $\mu$ M) for 48 h under 5% CO<sub>2</sub> and 100% humidity at 37° C. The cells were incubated for an additional

48h at 37°C with the compounds. DMSO at 0.5% was used as negative control (solvent control). Cell viability were evaluated by the resazurin method, as described below.

#### 4.7.3.3. Evaluation of cell viability of human PBMC by resazurin assay

The cell viability assay was performed according to O'Brien et al. (2000), with modifications.[40] Resazurin is a blue dye and is weakly fluorescent until it is irreversibly reduced to pink and red fluorescent resorufin. It is used as an oxidation-reduction indicator in cell viability assays and its intensity is proportional to the number of viable cells in a culture (Riss et al., 2000) [64]. Briefly, after 48 hours of incubation with the extracts, 20 µL of the resazurin solution at 50 µg/mL per well was added. The plates were incubated in a CO<sub>2</sub> incubator at 37°C for 3 hours. The fluorescence reading was performed at two wavelengths of excitation 530 and emission of 590 nm - in a plate reader (VarioScan, Thermo Scientific®). The number of viable cells correlates with the percentage of reduction of resazurin and were expressed as percent viability/proliferation as follows:

% cell viability: (fluorescence of the sample-blank) x100 / (fluorescence solvent control)

A blank of each sample was performed to avoid unspecified reactions of the compounds with resazurin (blank) and the results were analyzed using Prism 7.0 (GraphPad Software Inc).

#### 4.7.4. Selectivity index (SI) determination

After determining the IC<sub>50</sub> values for tumor (HL60, Jurkat, THP-1, MCF-7, MDA-MB-231 and HCT-116 cells) and non-tumor cells (PBMC), the selectivity index was calculated. Determination of the SI was performed by the ratio between IC<sub>50</sub> of PBMC and IC<sub>50</sub> tumor cell [65].

#### 4.7.5. DNA fragmentation assay (subdiploid DNA content)

The DNA fragmentation were analyzed according to Marques et al., (2020) [20]. Briefly, the cells (HL60, Jurkat, THP-1, MCF-7, MDA-MB-231 and HCT-116) were seed at density of 2x10<sup>5</sup> cell/well in 24-well plate and incubated overnight. Subsequently, the cells were treated with compounds **5**, **14-16**, etoposide and lapachol at 50µM (IC<sub>80</sub> value) for 24 hours. After treatment, the cells and the supernatant were collected and centrifuged at 500 rpm for 5 min in a micro-centrifuge (Denver Instrument Company, USA). The supernatant was discarded and the cells labelled with 300µL of a Hypotonic Fluorochromic

Solution containing 50 µg/mL propidium iodide and 0.1% Triton X-100 in 0.1% sodium citrate. After 2 h of incubation at 8 °C the samples were analyzed in flow cytometry (Becton–Dickinson, Mountain View, CA). A total of 10.000 events were acquired using Cell Quest and analyzed using FlowJo 7.6.4® (Tree Star, Inc.) to calculate the number of cells with fragmented DNA (sub-G0/G1).

#### *4.7.6. Clonogenic assay*

The MCF-7, MDA-MB-231 and HCT-116 cells were seed in 6-well plates at a density of 400 cells/well. After 6 hours of incubation, cells were treated with the compounds (at their IC<sub>50</sub> and IC<sub>80</sub>) or control (DMSO 0.5%). The cells were incubated with the compounds for 24 hours and then the medium was removed and replaced by supplemented DMEM medium without the compounds [66]. The cells were incubated for another 14 days and after incubation, the colonies were fixed in 70% alcohol for 15 minutes, stained with crystal violet (30% in ethanol) for 30 minutes and kept at room temperature overnight for drying. Colonies with 50 or more cells were counted. The survival fraction (ratio between the number of colonies treated with the compounds and the number of colonies counted in the control) were calculated and the results were analyzed by GraphPad Prism 7.0.

#### *4.7.6. Statistical analysis*

Data are expressed as the means± SD (standard deviation). Statistical analysis was conducted using the Prism 7.0 statistical package (GraphPad Software, USA). To ascertain significance, we used a one-way ANOVA with Bonferroni post-test. Statistical significance was considered at a limit of p<0.05 from three independent experiments conducted in triplicate.

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#### **Conflict of interest**

The authors declare no conflict of interest.

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