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Cytotoxic, immunomodulatory, antimycotic, and antiviral activities of semisynthetic 14-hydroxyabietane derivatives and triptoquinone C-4 epimers†

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A series of C14-hydroxy derivatives of dehydroabietic acid were synthesised from commercial abietic acid and evaluated for their cytotoxic, antimycotic, and antiviral activities. From these C14-hydroxy derivatives, triptoquinone C-4 epimers were obtained and their immunomodulatory activity was additionally evaluated. None of the tested compounds showed antiviral activity against herpes simplex virus type 1 (HHV-1), and nor did they display antimycotic activity against certain *Aspergillus*, spp. except for one compound, abieta-8,11,13-trien-14,18-diol. Interestingly, two triptoquinone epimers showed cytotoxic activity, and one of them induced mitochondrial potential loss, DNA damage and cell cycle distribution alterations in Jurkat cells, but not in human peripheral blood mononuclear cells. In addition, these compounds inhibited monocyte's differentiation and production of pro-inflammatory cytokines, IL-1β and TNF-α, and the anti-inflammatory cytokine IL-10 in the presence of LPS. In conclusion, one of the triptoquinone molecules could be a promising scaffold for the development of novel anti-cancer agents, and two of them could be potential anti-inflammatory agents.

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1 Introduction

Natural abietane phenols and quinones, as well as other oxidised related compounds, constitute an interesting group of diterpene metabolites, due to the significant biological activities exhibited by some of them. For example, ferruginol (**1**) (Fig. 1) presents interesting biological activities, such as antifungal and antitumor properties.[1]

Another interesting phenol abietane diterpene is triptinine B (**2**), which displays leukotriene D_4 antagonism.[2] Among the abietane quinones, representative examples are the antitumor and antimicrobial taxodione (**3**),[3–5] the antileishmanial agent 12-deoxyroyleanone (**4**),[6] and the antifungal and cytotoxic cryptoquinone (**5**).[7] Other significant quinones are a group of A-ring functionalised diterpenoids such as triptoquinones A–F (**6–11**), which are potent interleukin-1 inhibitors, and triptoquinone A, which also suppresses inducible nitric oxide synthase mRNA expression mediated by bacterial lipopolysaccharides.[8–10] Despite the diverse biological properties of this family of compounds, little effort has been made for their biological study, especially the cytotoxicity of derivatives towards tumour and non-tumour cells.

As a part of our research program towards the discovery of bioactive terpene-based compounds, we were interested in the biological study of some recently synthesised 14-hydroxyabietanes and their corresponding quinones obtained by

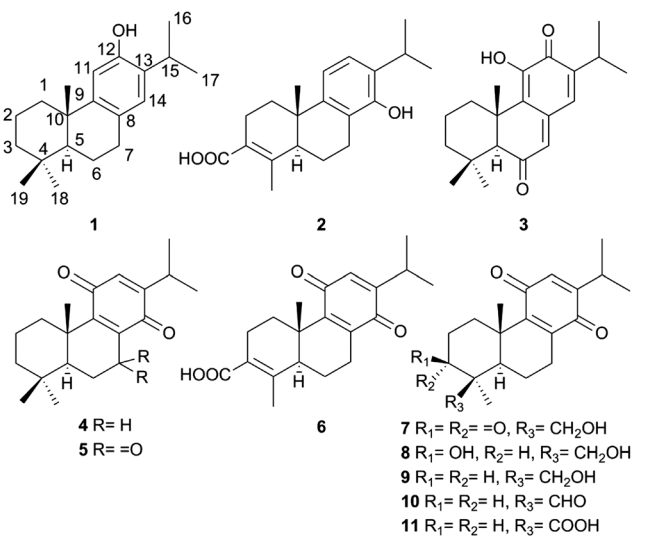

Fig. 1 Bioactive oxidised abietane phenols and quinones.

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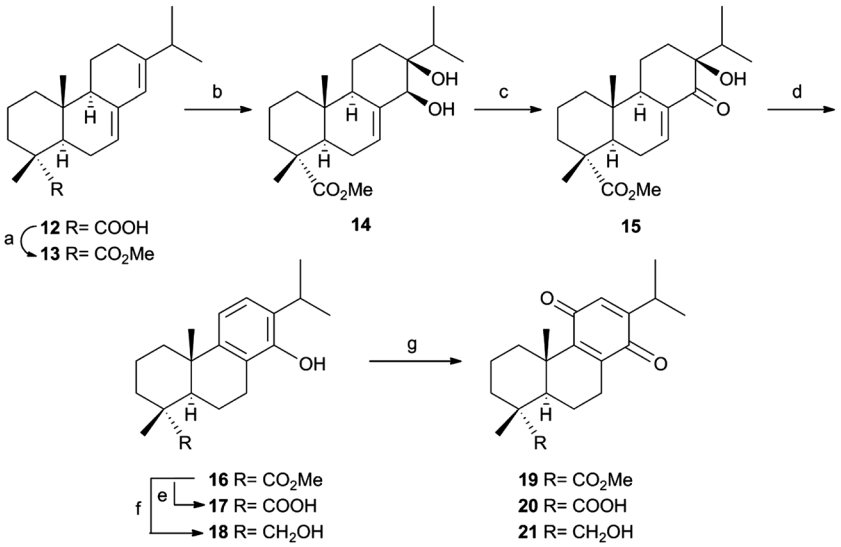

Scheme 1 *Reagents and conditions*: (a) LiOH, Me_2SO_4, DMF; (b) OsO_4, Me_3NO, py, *t*-BuOH; (c) PhSeSePh, *t*-BuOH, CCl_4; (d) TsOH, toluene, reflux; (e) KOH, MeOH, H_2O; (f) $LiAlH_4$ and THF; (g) H_2O_2, $RuCl_3$, AcOH.

oxidation.[11,12] These quinones represent C-4 epimers of natural triptoquinones the biological activity of which has not yet been studied. Here, we describe the syntheses of a number of abietane derivatives from commercially available (−)-abietic acid (**12**),[13] and the evaluation of their cytotoxic, antimycotic, antiviral and immunomodulatory activities. In this study, an oxygenated moiety (such as methyl ester, alcohol, or acid) was introduced into the lipophilic abietane skeleton. Compound **16** and five derivatives (**17–21**) with different functional groups at C14 and C18 were tested (Scheme 1).

2 Results

2.1 Chemistry

The synthesis of the C18-functionalised dehydroabietanes used in this work begins with the preparation of the key intermediate 14-hydroxydehydroabietic acid or related compounds from commercial (−)-abietic acid as outlined in Scheme 1. Firstly, abietic acid **12** was esterified by treatment with lithium hydroxide and methyl sulfate to yield ester **13** in a quantitative yield.[14] Then, regioselective dihydroxylation of **13** gave diol **14** in a 70% yield. Oxidation of the allylic alcohol in **14** with PhSeSePh and *t*-BuOOH in CCl_4 gave the hydroxy ketone **15** in a 75% yield. Subsequent aromatisation of **15** with TsOH in refluxing toluene provided the desired phenol (methyl 14-hydroxydehydroabietate) **16** in an 80% yield.

With phenol **16** in hand, we carried out the change of functionalization at C-18 and, finally, the reaction of oxidation with H_2O_2, and $RuCl_3$ as a catalyst, to yield the corresponding quinones. Thus, the methyl ester group in **16** was saponified with KOH in aqueous methanol to yield acid **17**. Reduction of ester **16** with $LiAlH_4$ in dry tetrahydrofuran at reflux gave alcohol **18**. Finally, phenol oxidation of **16–18** with H_2O_2, in the presence of cat. $RuCl_3$, supplied quinones **19–21** in a moderate yield. The oxidation of the primary alcohol in **18** and **21** with Dess–Martin periodinane failed to yield the corresponding aldehydes.

2.2 Biological evaluation

2.2.1 Antiviral activity. The antiviral activity of 14-hydroxyabietane (phenols) (**16–18**) and triptoquinone epimers (**19–21**) against herpes simplex virus type 1 (HHV-1) was determined using a modified end-point titration technique (EPPT) according to the protocol reported by us.[15] None of the tested compounds reduced the HHV-1 replication at the evaluated concentrations (data not shown).

2.2.2 Antimycotic activity. Compounds **16–21** were tested *in vitro* for antimycotic activity. The compounds did not show antimycotic activity against *Aspergillus fumigatus*, *A. flavus*, *A. niger*, and *A. terreus* in concentrations below 100 μg mL^{-1}, except for the 14-hydroxyabietane derivative **18**, which showed a MIC value of 25 μg mL^{-1} against *A. fumigatus* and *A. terreus*, and 50 μg mL^{-1} against *A. niger* (data not shown); however, it showed cytotoxic activity against Vero cells. The MIC value for the reference antifungal drug, amphotericin B (Sigma, New Jersey, USA), used as a positive control, was within the established values for the CLSI-M38-A protocol.

2.2.3 Cytotoxic activity: MTT assay. We determined the concentration for each compound that inhibited 50% of Jurkat, Vero and HeLa cells growth (IC_{50}) at 48 h through a microculture tetrazolium (MTT) assay.

All the tested compounds (**16–21**) produced a dose-dependent inhibition of the growth of the Jurkat and HeLa tumor cell lines and the Vero cell line, with an R^2 (coefficient of linear regression) > 0.8. The triptoquinone epimers **20** (C-4 epimer of triptoquinone F) and **21** (C-4 epimer of triptoquinone D) showed the lowest IC_{50} values on the Jurkat tumor cell line corresponding to 14.4 ± 2.4 μg mL^{-1} and 14.6 ± 3.8 μg mL^{-1}, respectively (Table 1).

Table 1 IC_{50} values (μg mL^{-1}) of 14-hydroxyabietane and triptoquinone epimers **16–21** on Jurkat, HeLa and Vero cell lines

	Cell lines[c]				
	Jurkat		HeLa		Vero
Compound	IC_{50}[a]	SI[b]	IC_{50}	SI	IC_{50}
16	14.2 ± 4.2	2.59	18.6 ± 4.0	1.90	36.8 ± 6.2
17	34.0 ± 6.9	3.09	136.4 ± 2.1	0.77	105.2 ± 11
18	8.2 ± 1.2	2.30	26.6 ± 5.8	0.71	19.0 ± 4.6
19	18.5 ± 4.3	2.34	35.2 ± 1.9	1.23	43.3 ± 5.2
20	14.4 ± 2.4	8.96	27.2 ± 5.0	4.74	129.0 ± 6.3
21	14.6 ± 3.8	5.46	79.5 ± 16.0	1.00	79.7 ± 15.6

[a] Concentration of compounds that induces 50% growth inhibition in 48 h. [b] SI, selectivity index is defined as Vero IC_{50} over either Jurkat or HeLa IC_{50}. [c] Jurkat, human acute T cell leukemia ATCC TIB-152; HeLa, human cervix epitheloid adenocarcinoma cells ATCC CRL-1958; Vero, *Cercopithecus aethiops* African green monkey kidney cells ATCC CCL-81.

Compounds **16** and **18** showed the lowest IC_{50} values on the HeLa cell line, corresponding to 18.6 ± 4.0 μg mL^{-1} and 26.6 ± 5.8 μg mL^{-1}, respectively (Table 1). However, the selectivity index (SI) of these compounds on HeLa was <5. Compounds **20** and **21** showed the lowest IC_{50} values and the highest selectivity index on the Jurkat cell line; therefore, their possible cytotoxic mechanism towards the Jurkat tumor cell line as well as the selectivity of their mechanism using PBMC as a non-tumor cell line control were evaluated.

2.2.4 Lymphoproliferative responses evaluation on PBMCs. Our preliminary data showed that compounds **20** and **21** could exhibit anti-tumour activity against acute T cell leukemia (Jurkat), but it is necessary to prove the effect on non-tumor human cells. Therefore, the lymphoproliferative activity of compounds **20** and **21** on PBMCs has also been evaluated. Moreover, we evaluated if the cytotoxic effect of compounds **20** and **21** on Jurkat cells is through the inhibition of cellular proliferation. Lymphocyte proliferation by serial halving of the fluorescence intensity of the vital dye CFSE has become widely used. We calculated the percentage of inhibition of cell proliferation caused by each compound, through the comparison of the CFSE fluorescence intensity (MFI) of cells treated to the MFI of untreated cells.

Flow cytometry analysis showed that the triptoquinone epimer **21** inhibited 77% and 82% of proliferation of Jurkat cells at concentrations of 7.0 μg mL^{-1} and 14.0 μg mL^{-1}, respectively. In contrast, the triptoquinone epimer **20** did not inhibit Jurkat cells proliferation at the evaluated concentrations (Fig. 2a, see ESI†).

The CFSE assay has significant advantages in terms of the ability to gate specific populations of lymphocytes. Then, the effect of triptoquinone epimers on proliferation of CD4 and CD8 cells stimulated with PHA was evaluated. The percentage of proliferation of CD4 and CD8 cells was calculated comparing the percentage of proliferation of the treated CD4 or CD8 cells to the total untreated cells CD4 or CD8 (100%). Triptoquinone epimers **20** and **21** inhibited proliferation of both CD4 and CD8 cells at concentrations of 7.0 μg mL^{-1} and 14.0 μg mL^{-1} after 72 h of treatment. In addition, the inhibition of proliferation of CD4 and CD8 cells was higher after treatment with the triptoquinone epimer **21** at the concentration of 14.0 μg mL^{-1}, corresponding to 88% and 77% of inhibition, respectively (Fig. 2b, see ESI†). The results indicate that the effect on proliferation inhibition of triptoquinone epimers **20** and **21** was not specific for either CD4 or CD8 cells.

In order to study whether the proliferation inhibition induced by triptoquinone epimers **20** and **21** was due to inhibition of activation induced by PHA, the PBMCs were treated one hour after or one hour before adding PHA and then evaluated for the expression of the early lymphocyte activation marker CD69.

Both compounds **20** and **21** did not induce reduction in the percentage of activated CD4 or CD8 cells stimulated one hour before or after treatment at concentrations of 1, 7.0 and 14.0 μg mL^{-1}, as compared to control cells (data not shown). These results suggest that both compounds inhibited neither proliferation nor stimulation induced by PHA.

2.2.5 Evaluation of mitochondrial potential and integrity of the membrane. Considering that one of the reported effects of antitumor quinone-based compounds, such as 2-methylnaphtho[2,3-*b*]furan-4,9-dione, is the induction of a loss in the mitochondrial potential in tumor cell lines,[16,17] the effect of triptoquinone epimers **20** and **21** on Jurkat cells and PBMC mitochondrial potential was evaluated using ($DiOC_6$), and also the membrane integrity was evaluated using PI. The triptoquinone epimer **21** induced a loss of the mitochondrial membrane potential in Jurkat cells at a concentration of 14.0 μg mL^{-1} after 15 h and 24 h of treatment, corresponding to 15% and 25%, respectively (Fig. 3, see ESI†); this effect was not observed at a concentration of 7.0 μg mL^{-1}. In contrast, the triptoquinone epimer **20** did not induce a loss of the mitochondrial membrane potential in Jurkat cells or PBMCs at the evaluated concentrations (Fig. 3a, see ESI†). In PBMCs, the triptoquinone epimer **21** induced a loss of the mitochondrial membrane potential at a concentration of 14.0 μg mL^{-1} after 15 h or 24 h of treatment corresponding to 20% and 90%, respectively (Fig. 3b, see ESI†). None of the compounds altered the membrane integrity in PBMCs or Jurkat cells (data not shown).

2.2.6 Evaluation of phosphatidylserine exposure. Taking into account that the loss of the mitochondrial membrane potential is an event that precedes several forms of cell death, we verified whether apoptosis was occurring. To this end, Jurkat and PBMC cells were stained with Annexin V and counterstained with PI, and then analyzed by means of flow cytometry. The viable cells, early apoptotic cells, late apoptotic cells and necrotic cells are shown in Fig. 4 and 5 (see ESI†). An increase of Annexin V+ Jurkat cells was observed after 15 h and 24 h of treatment with compounds **20** and **21** at a concentration of 14.0 μg mL^{-1}. Additionally, the triptoquinone epimer **21** induced the highest increase in the percentage of Annexin V+ Jurkat cells, corresponding to 26% and 86% with respect to the control, after treatment for 15 h and 24 h, respectively (Fig. 4, see ESI†). Similarly, after the treatment of PBMCs with triptoquinone epimers **20** and **21** at a concentration of 14.0 μg mL^{-1}

for 15 h and 24 h, the percentage of positive Annexin cells increased in comparison to the control. The triptoquinone epimer **21** induced the highest increase in the percentage of Annexin V+ PBMCs corresponding to 75% and 90% in comparison to the control, after treatment for 15 h and 24 h, respectively (Fig. 5†). These results indicate that compounds **20** and **21** induce phosphatidylserine exposure on Jurkat and PBMC cells. Compound **21** induced the highest phosphatidylserine exposure.

2.2.7 Evaluation of cell cycle distribution. We also investigated whether compounds **20** and **21** induced either DNA damage or alteration in the cell cycle distribution of Jurkat and PBMC cells, using flow cytometry analysis. After treating Jurkat cells with the triptoquinone epimer **21** for 15 h and 24 h, there was a marked increase in the percentage of cells in the G2/M-phase of the cell cycle, corresponding to 13% and 15%, respectively, accompanied by a decrease in the percentage of cells in the G0/G1-phase. In contrast, the triptoquinone epimer **20** did not alter the cell cycle distribution of Jurkat cells (Fig. 6, see ESI†).

The triptoquinone epimer **21** induced hypoploidy in Jurkat cells at a concentration of 14.0 μg mL^{-1}, after 15 h and 24 h of treatment, corresponding to 12% and 35%, respectively, while the triptoquinone epimer **20** did not induce hypoploidy at the evaluated concentrations (Fig. 7, see ESI†). In stimulated PBMCs, triptoquinone epimers **20** and **21** did not induce alteration of the cell cycle distribution, and did not cause hypoploidy at the tested concentrations (data not shown).

2.2.8 TUNEL assay. To prove that the epimer **20** did not induce DNA damage in Jurkat and PBMC cells in comparison to the epimer **21**, we carried out a TUNEL assay. The triptoquinone epimer **21** induced an increase of positive TUNEL Jurkat cells at a concentration of 14.0 μg mL^{-1}, after 15 h and 24 h of treatment, corresponding to 10% and 35%, respectively, but did not induce an increase in PBMCs at the evaluated concentrations (Fig. 8b†). In contrast, the triptoquinone epimer **20** did not induce any DNA damage in Jurkat or PBMC cells at the evaluated concentrations (Fig. 8, see ESI†). These results are in agreement with the results of staining with PI that were described above (Fig. 7, see ESI†).

2.2.9 Evaluation of mononuclear phagocyte differentiation. As triptoquinone D and triptoquinone F are potent interleukin-1α and interleukin-1β inhibitors,[8] we evaluated the anti-inflammatory activity of their C-4 epimers, compounds **20** and **21** respectively, in lipopolysaccharide (LPS)-stimulated monocyte-derived macrophages. Initially we investigated the effect of triptoquinone epimers **20** and **21** on the differentiation of monocytes into macrophages measuring the expression of HLA-DR and fluorescein diacetate labeling (FDA) after 120 h of culture.

Both compounds induced a reduction in HLA-DR expression (Fig. 9a, see ESI†) and MFI of FDA (Fig. 9b, see ESI†) after treatment at concentrations of 5.0, 10.0 and 15.0 μg mL^{-1}, as compared to control cells.

2.2.10 Cytokine production by monocytes. The effect of triptoquinone epimers **20** and **21** on cytokine production, in the presence or absence of LPS, was evaluated. Both compounds reduced the production of IL-1β, TNFα and IL-10 in PBMCs stimulated with LPS after treatment at concentrations of 5.0, 10.0 and 15.0 μg mL^{-1} (Fig. 10, see ESI†), and there were no significant differences in inhibition of cytokine production between compounds, except for IL-1β, which was more inhibited by the triptoquinone epimer **20** at concentrations of 5.0 and 10.0 μg mL^{-1}. The level of cytokines did not differ in the absence of LPS after treatment with both compounds (data not shown).

3 Discussion

The present study was performed to evaluate the antimycotic, antiviral and cytotoxic activity of three semisynthetic triptoquinone epimers and three semisynthetic 14-hydroxyabietane derivatives. None of the tested compounds showed relevant antiviral or antimycotic activity, however some of them showed cytotoxic activity.

Compound **16** showed the lowest IC_{50} value on the HeLa cell line, but the selectivity index of this compound was <5. In the Jurkat tumor cell line, compounds **20** and **21** inhibited cellular growth and showed the highest selectivity index (SI) values >5, corresponding to 8.96 and 5.46, respectively.

Triptoquinone epimers (**19–21**) were more selective to Jurkat than HeLa cells, especially compound **20**, and this is possibly due to the presence of a molecular target in Jurkat that is not present in HeLa cells.

According to the National Cancer Institute (USA), a crude extract is cytotoxic when its IC_{50} on normal cells is below 30 μg mL^{-1};[18] therefore compounds **20** and **21**, with IC_{50} values of 129.0 ± 6.3 and 79.7 ± 15.6, respectively, are not cytotoxic in Vero cells. So they were selected to evaluate their possible mechanism of cell death induction.

Here, it was demonstrated that only the triptoquinone epimer **21** induced proliferation inhibition in the Jurkat cell line when it was evaluated for the vital dye carboxyfluorescein diacetate succinimidyl (CFSE) assay. In addition, in order to identify the selectivity on proliferation inhibition of compounds **20** and **21** in tumor-lines, their effect on cell proliferation in PBMCs was determined. Both compounds **20** and **21** affected CD4 or CD8 cells proliferation after 72 h of treatment, but not their activation (CD69 expression), which is very important considering that CD69 is a co-stimulatory molecule for T-cell activation after stimulation through the T cell antigen receptor (TCR) as well as a signal-transmitting receptor for synthesis of cytokines (IL-2, INF-α, and TNF-α), expression of the IL-2R subunit, rise of intracellular calcium concentration and cell proliferation necessary for immune response.[19]

Desmond *et al.* (2005) have reported the ability of the furanonaphthoquinones to cause growth arrest and apoptosis in a variety of human leukemia and multiple myeloma cell lines;[16] these effects were associated with a decrease in mitochondrial function that mediates the release of some proapoptotic proteins. In this study, it was found that only the triptoquinone epimer **21** induced a loss of the mitochondrial membrane potential in both Jurkat cells and PBMCs at a concentration of 14 μg mL^{-1} but not at a concentration of 7 μg mL^{-1}, suggesting

that at lower concentrations, it can be more selective to Jurkat cells. Furthermore, none of the triptoquinone epimers altered the membrane integrity in PBMCs or Jurkat cells. This suggests that triptoquinone epimers **20** and **21** do not induce necrosis, which is important because pharmacological-induced necrosis might lead to tissue damages.[20]

Alteration of cell cycle distribution was only observed with compound **21**, which was an increase in the percentage of cells in the G2/M-phase and a decrease in the percentage of cells in the G0/G1-phase.

Gomathinayagam *et al.* (2008) observed that "Plumbagin", a naphthoquinone, present in plants from the *Droseraceae* and *Plumbaginaceae* families, caused an increase in the percentage of cells in the G2/M-phase in human lung cancer cells (H460) by down-regulating G2/M regulatory proteins (cyclinB1 and Cdc25B), and it also induced a decrease in the percentage of cells in the G0/G1-phase.[21]

Likewise, only compound **21** induced selective DNA damage in Jurkat cells at the tested concentrations. It is noteworthy to say that this has been observed with another diterpenoid quinone, "Salvicine", which induces DNA damage through double-strand breaks by inhibition of topoisomerase II and glutathione depletion (Cai *et al.*, 2008).[22] In contrast, diterpenoid quinones isolated from the plant *Peltodon longipes* and that possess a *para*-quinone structure such as 7-α-acetoxyroyleanone, horminone, royleanone, and 7-ketoroyleanone have shown cytotoxic activity against the human pancreatic cancer cell line MIA PaCa-2, mainly through the inhibition of the relaxation activity of human topoisomerase I (induced by DNA strand breaks) at concentrations lower than or similar to those of the positive control, camptothecin.[23] As said by Fronza *et al.* (2012),[23] it is possible that the structural properties of *para*-quinone diterpenes can influence or determine their molecular mode of producing cell death. Therefore, these different modes of action suggest that cell death induced by abietane quinone diterpenes may not follow a single mechanism, but several mechanisms instead.

Apoptotic cell death is a process of programmed cell death that includes several biochemical events, among others, protein cleavage, protein cross-linking, DNA breakdown and loss of the mitochondrial membrane potential. Wong *et al.* (2012) have observed that Tanshinone IIA (Tan IIA), a diterpenoid naphthoquinone derived from *Radix salvia miltiorrhiza*, induces apoptosis in lung cancer cells (A549) and human colon adenocarcinoma cells (Colo-205) through a mitochondrial mediated intrinsic cell death pathway.[24–26] Tan IIA has shown cytotoxic activity against human prostate cancer cells (LNCaP) at a concentration of 35 μM and induces a reduction of the mitochondrial membrane potential at a concentration of 50 μM.[24]

In this study, the externalization of phosphatidylserine residues in the outer plasma membrane, an early biochemical event that occurs in apoptotic cells, was also evaluated. It was observed that both compounds **20** and **21** induced phosphatidylserine (Anexin V) exposure on both PBMCs and Jurkat cell line at a tested concentration of 14.0 μg mL^{-1} and compound **21** induced the highest percentage of positive Annexin cells in both PBMCs and Jurkat cell line.

The triptoquinones A–F (**6–11**)(Fig. 1) are principal components of *Triptergium wilfordii* and have been found to inhibit production or release of IL-1 by human monocytes stimulated by endotoxin.[9,27,28] Then, the effects of compounds **20** and **21** on differentiation of monocytes were investigated and it was found that both compounds interfered with monocyte differentiation as assessed by HLA-DR expression and FDA labeling.

The inhibition of production or release of IL-1β in rat thoracic aorta after LPS treatment has been shown by the triptoquinone A.[8] In addition, *T. wilfordii* extracts and their active compounds, tryptoquinones D and F, have also showed inhibition of IL-1β production in human monocytes[28] and IL-1β activity in human peripheral mononuclear cells.[9] Takaishi *et al.* (1997) observed that triterpenoids isolated from *T. wilfordii* var. *Regelii* have inhibitory activity against IL-1α and IL-1β release from lipopolysaccharide-stimulated human peripheral mononuclear cells.[27]

Several studies have reported that Triptolide, a purified diterpenoid component of *T. wilfordii*, is able to suppress the production of inflammatory mediators such as IL-1β and TNF-α induced by LPS in various cell types including bronchial epithelial cells,[24,29] peripheral blood mononuclear cells,[30,31] and macrophages.[32]

Several studies suggest that the *T. wilfordii* bioactive compounds (triptolide, celastrol and tripchlorolide) exert their immunosuppressive and anticancer activities by modulating the transcriptional activity of the nuclear factor-NF-κB. NF-κB activates the enhancer region of various genes, including the pro-inflammatory cytokines tumour necrosis factor (TNF)-α and oncogenic genes, such as survivin, cyclinD1 and c-myc.[24] Additionally, several clinical trials have shown the efficacy of Triptolide in the *T. wilfordii* extracts in the treatment of rheumatic diseases, including rheumatoid arthritis and systemic lupus erythematosus.[24,33–37]

Based on this evidence, the anti-inflammatory effect of triptoquinone epimers **20** and **21**was evaluated. Both compounds not only inhibited the production of IL-1β and TNF-α, pro-inflammatory cytokines, but also IL-10 an anti-inflammatory cytokine in the presence of LPS. Therefore the compounds did not possess a differential effect on the production of pro-inflammatory and anti-inflammatory cytokines.

4 Conclusions

In summary, the triptoquinone epimers **20** and **21** showed different effects and selectivity, which could be due to the difference in their chemical structure; both compounds differ only in one of their substitutions on carbon 18. Compound **20** has a carboxylic acid as the functional group and compound **21** has an alcohol. In addition, the triptoquinone epimer **20** induced only phosphatidylserine exposure on Jurkat cells, therefore, it was not clear if this epimer induces cell death. On the other hand, the triptoquinone epimer **21** induced phosphatidylserine exposure and proliferation inhibition in PBMCs but it did not affect their activation. Moreover, it induced a loss of the mitochondrial potential at a concentration of 7.0 μg mL^{-1}, DNA damage and alteration of cell cycle distribution in Jurkat cells but not in PBMCs. These results suggest that this compound induces cell death in the Jurkat tumoral cell line at a

concentration of 7.0 μg mL^{-1}, however, *in vivo* studies will be necessary to define its possible clinical usefulness.

In addition, taking into account that both compounds inhibited monocyte differentiation, they could be potential immunosuppressive and anti-inflammatory agents that can be useful in treatment of autoimmune diseases such as rheumatoid arthritis and non-infectious inflammatory processes.[38]

5 Materials and methods

5.1 Chemistry

Optical rotations were determined with a 5 cm path-length cell, using dichloromethane as a solvent (concentration expressed in g per 100 mL). $[\alpha]_D$-values are given in 10^{-1} deg cm^2 g^{-1}. NMR spectra were recorded on a 300 MHz spectrometer with tetramethylsilane as an internal standard. All spectra were recorded in $CDCl_3$ as the solvent unless otherwise described. Complete assignments of ^{13}C NMR multiplicities were made on the basis of DEPT experiments. *J* values are given in Hz. Mass spectra (MS) were run by electron impact (EI) at 70 eV. Reactions were monitored by means of thin-layer chromatography (TLC) using Merck silica gel 60 F-254 in 0.25 mm thick plates. Compounds on TLC plates were detected under UV light at 254 nm and visualized by immersion in a 10% sulfuric acid solution and heating on a hotplate. Purifications were performed by flash chromatography on Merck silica gel (230–400 mesh). All non-aqueous reactions were carried out in an argon atmosphere in oven-dried glassware. Commercial reagent grade solvents and chemicals were used as received unless otherwise noted. Combined organic extracts were washed with brine, dried over anhydrous sodium sulphate, filtered and concentrated under reduced pressure.

5.1.1 Methyl 13β,14β-dihydroxyabieta-7-en-18-oate (14). To a solution of methyl abietate (**13**) (González *et al.*, 2009)[14] (6.0 g, 0.018 mol) in *t*-BuOH (30 mL) and pyridine (1.4 mL), Me_3NO (2.8 g, 1.3 equiv.) and a 4% solution of OsO_4 (2.0 mL) were added. The reaction mixture was stirred under an Ar atmosphere at reflux for one week. Then, $NaHSO_3$ (5 mL) was added and the solvent was evaporated. The residue was dissolved in AcOEt and washed with brine. The organic layer was dried and concentrated to yield the crude product which was purified by chromatography on silica, eluted with hexane–ethyl acetate (from 7 : 3 to 6 : 4) to yield diol 14 (4.6 g, 70%) as an orange oil that solidifies upon standing. The MS, ^1H and ^{13}C data are in agreement with the literature data.[39]

5.1.2 Methyl 13β-hydroxy-14-oxoabieta-7-en-18-oate (15). Diphenyl diselenide (6.2 g, 20 mmol) and 5.5 M *t*-BuOOH in decane (7.3 mL, 40 mmol) were added to a stirred solution of **14** (5.0 g, 14.3 mmol) in dry CCl_4 (100 mL), and the mixture was refluxed under an Ar atmosphere for 4 h. Then, the solvent was evaporated under vacuum, and the residue was purified by chromatography on silica, eluted with hexane–diethyl ether (7 : 3) to yield ketone 15 (3.7 g, 75%) as a colourless oil. The MS, ^1H and ^{13}C data are in agreement with the literature data.[40]

5.1.3 Methyl 14-hydroxyabieta-8,11,13-trien-18-oate (16). To a solution of the ketone **15** (2.0 g, 5.7 mmol) in toluene (100 mL), *p*-toluenesulfonic acid (1.1 g, 5.7 mmol) was added and the mixture was refluxed for 15 h. Then, the solvent was evaporated to yield a residue, which was chromatographed on silica, eluted with hexane–ethyl acetate (8 : 2) to yield phenol **16** (1.5 g, 80%) as an orange oil: $[\alpha]_D^{25}$ +36.0 (c 4.5) [(ref. 8) +51.7 (c 0.5, $CHCl_3$); (ref. 9) +7.7 (c 1.0, $CHCl_3$)]. ^1H NMR (300 MHz) δ 7.00 (1H, d, $J = 8.2$), 6.82 (1H, d, $J = 8.2$), 3.64 (3H, s), 3.16 (1H, sept., $J = 6.9$), 2.77 (1H, dd, $J = 16.8, 6.3$), 2.67 (1H, m), 2.27 (1H, br d, $J = 12.9$), 2.21 (1H, dd, $J = 12.6, 2.1$), 1.27 (3H, s), 1.21 (6H, d, $J = 6.9$), 1.20 (3H, s); ^{13}C NMR (75 MHz) δ_C 179.0 (s), 150.3 (s), 148.0 (s), 130.3 (s), 123.1 (d), 120.7 (s), 115.9 (d), 51.7 (d), 47.4 (s), 44.0 (d), 37.9 (t), 36.7 (s), 36.4 (t), 26.5 (q), 24.8 (q), 23.9 (t), 22.6 (q), 22.4 (q), 20.9 (t), 18.4 (t), 16.3 (q); HRMS (EI) *m*/*z* 330.2204 $[M]^+$, calcd for $C_{21}H_{30}O_3$: 330.2195.

5.1.4 14-Hydroxyabieta-8,11,13-trien-18-oic acid (17). A mixture of ester **16** (200 mg, 0.61 mmol), KOH (85%, 1.5 g, 23 mmol), H_2O (2 mL) and methanol (12 mL) was refluxed for 48 h. After this time, the reaction mixture was cooled, poured into aqueous HCl (1.2 M, 30 mL) and extracted three times with DCM. The organic extract was dried over $MgSO_4$ and concentrated under reduced pressure to yield the crude acid **17**, which was chromatographed on silica, eluted with hexane–ethyl acetate (6 : 4) to yield acid **17** (144 mg, 75%) as a yellow foam: $[\alpha]_D^{25}$ +46.4 (c 1.2) [(ref. 8) +58.9 (c 0.5, $CHCl_3$)]. ^1H NMR (300 MHz) δ 7.01 (1H, d, $J = 8.1$), 6.84 (1H, d, $J = 8.1$), 3.14 (1H, sept., $J = 6.9$), 2.76 (1H, dd, $J = 16.8, 6.6$), 2.68 (1H, m), 2.28 (1H, d, $J = 12.6$), 2.21 (1H, d, $J = 11.1$), 1.27 (3H, s), 1.22 (6H, d, $J = 6.9$), 1.20 (3H, s); ^{13}C NMR (75 MHz) δ_C 185.2 (s), 150.1 (s), 148.1 (s), 130.4 (s), 123.3 (d), 120.7 (s), 116.2 (d), 47.3 (s), 43.9 (d), 37.9 (t), 36.8 (s), 36.6 (t), 26.7 (q), 25.0 (q), 23.9 (t), 22.7 (q), 22.5 (q), 21.0 (t), 18.5 (t), 16.1 (q); HRMS (EI) *m*/*z* 316.2045 $[M]^+$, calcd for $C_{20}H_{28}O_3$: 316.2038.

5.1.5 Abieta-8,11,13-trien-14,18-diol (18). To a solution of ester **16** (500 mg, 1.51 mmol) in dry THF (10 mL) $LiAlH_4$ (500 mg, 13 mmol) was added in portions, and then refluxed for 15 h. Then, the mixture was cooled to 0 °C, and 0.5 mL of H_2O, 0.5 mL of 15% NaOH and 1.5 mL of H_2O were added sequentially and carefully. The resulting white solid was filtered off and washed with ethyl acetate. The extract was concentrated and purified by means of chromatography, eluted with hexane–ethyl acetate (6 : 4) to yield 380 mg (83%) of pure alcohol **18** as a yellowish foam: $[\alpha]_D^{25}$ +32.6 (c 2.1) [(ref. 8) +51.4 (c 0.75, $CHCl_3$)]. ^1H NMR (300 MHz) δ 6.99 (1H, d, $J = 8.1$), 6.83 (1H, d, $J = 8.1$), 5.03 (1H, s, OH), 3.45 (1H, d, $J = 10.8$), 3.18 (1H, d, $J = 10.8$), 3.14 (1H, sept., $J = 6.9$), 2.76 (1H, dd, $J = 16.2, 5.7$), 2.62 (1H, m), 2.25 (1H, br d, $J = 12.9$), 1.23 (3H, d, $J = 6.9$), 1.21 (3H, s), 1.20 (3H, d, $J = 6.9$), 0.85 (3H, s); ^{13}C NMR (75 MHz) δ_C 150.2 (s), 148.6 (s), 130.2 (s), 123.0 (d), 120.8 (s), 116.2 (d), 71.9 (t), 42.9 (d), 38.4 (t), 37.6 (s), 37.2 (s), 34.9 (t), 26.7 (q), 25.2 (q), 23.9 (t), 22.7 (q), 22.5 (q), 18.6 (t), 18.1 (t), 17.3 (q); HRMS (EI) *m*/*z* 302.2257 $[M]^+$, calcd for $C_{20}H_{30}O_2$: 302.2246.

5.1.6 Methyl 11,14-dioxoabieta-8,12-dien-18-oate (19). To a solution of phenol **16** (90 mg, 0.27 mmol) in AcOH (1 mL) at 10 °C, a catalytic amount of $RuCl_3 \cdot 3H_2O$ (14 mg, 0.2 equiv.) and 30% H_2O_2 (150 μL, 1.42 mmol) were added. The mixture was allowed to warm to room temperature and stirred for 15 h. Then, the reaction mixture was carefully poured into saturated $NaHCO_3$ and extracted with ethyl acetate. The organic layer was

washed with brine, dried and concentrated. The residue was chromatographed on silica, eluted with hexane–ethyl acetate (8 : 2) to yield quinone **19** (56 mg, 60%) as a yellow-orange oil: $[\alpha]_D^{25}$ −43.6 (c 2.2) [(ref. 8) −69.7 (c 0.5, $CHCl_3$)]. ^{1}H NMR (300 MHz) δ 6.32 (1H, d, $J = 1.2$), 3.68 (3H, s), 2.98 (1H, m), 2.78 (1H, br d, $J = 11.1$), 2.65 (1H, dd, $J = 20.1, 6.0$), 2.40 (1H, dd, $J = 11.4, 7.5$), 1.99 (1H, dd, $J = 12.3, 1.8$), 1.31 (3H, s), 1.25 (3H, s), 1.11 (3H, d, $J = 6.9$), 1.09 (3H, d, $J = 6.9$); ^{13}C NMR (75 MHz) δ_C 187.8 (s), 178.6 (s), 152.9 (s), 150.0 (s), 142.6 (s), 131.8 (d), 52.0 (d), 47.7 (s), 45.7 (d), 37.8 (s), 36.4 (t), 35.5 (t), 26.3 (q), 25.4 (t), 21.3 (q), 21.2 (q), 20.3 (q), 20.2 (t), 18.1 (t), 16.6 (q); HRMS (EI) m/z 344.1977 $[M]^+$, calcd for $C_{21}H_{28}O_4$: 344.1988.

5.1.7 11,14-Dioxoabieta-8,12-dien-18-oic acid (20). To a solution of phenol **17** (90 mg, 0.28 mmol) in AcOH (1 mL) at 10 °C, a catalytic amount of $RuCl_3 \cdot 3H_2O$ (14 mg, 0.2 equiv.) and 30% H_2O_2 (150 μL, 1.42 mmol) were added. The mixture was allowed to warm to room temperature and stirred for 15 h. Then, the reaction mixture was poured into water and extracted with ethyl acetate. The organic layer was washed with brine, dried and concentrated. The residue was chromatographed on silica, eluted with hexane–ethyl acetate (6 : 4) to yield quinone **20** (50 mg, 55%) as a yellow-orange semi-solid: $[\alpha]_D^{25}$ −52.8 (c 2.5) [(ref. 8) −68.0 (c 0.5, $CHCl_3$)]. ^{1}H NMR (300 MHz) δ 6.33 (1H, d, $J = 1.0$), 2.98 (1H, m), 2.80 (1H, br d, $J = 12.9$), 2.67 (1H, dd, $J = 20.1, 4.8$), 2.00 (1H, d, $J = 11.1$), 1.31 (3H, s), 1.26 (3H, s), 1.10 (3H, d, $J = 6.9$), 1.09 (3H, d, $J = 6.9$); ^{13}C NMR (75 MHz) δ_C 187.8 (s), 187.7 (s), 152.9 (s), 149.9 (s), 142.7 (s), 131.8 (d), 45.4 (d), 37.7 (s), 36.5 (t), 35.5 (t), 26.3 (q), 25.4 (t), 21.3 (q), 21.3 (q), 20.3 (q), 20.2 (t), 18.1 (t), 16.3 (q); HRMS (EI) m/z 330.1792 $[M]^+$, calcd for $C_{20}H_{26}O_4$: 330.1831.

5.1.8 11,14-Dioxoabieta-8,12-dien-18-ol (21). To a solution of phenol **18** (250 mg, 0.82 mmol) in AcOH (2.5 mL) at 10 °C, a catalytic amount of $RuCl_3 \cdot 3H_2O$ (34 mg, 0.2 equiv.) and 30% H_2O_2 (450 μL, 1.42 mmol) were added. The mixture was allowed to warm and stirred for 15 h. Then, the reaction mixture was carefully poured into saturated $NaHCO_3$ and extracted with ethyl acetate. The organic layer was washed with brine, dried and concentrated. The residue was chromatographed on silica, eluted with hexane–ethyl acetate (8 : 2) to yield quinone **21** (150 mg, 58%) as an orange oil: $[\alpha]_D^{25}$ −36.5 (c 7.5) [(ref. 8) −64.1 (c 0.6, $CHCl_3$)]. ^{1}H NMR (300 MHz) δ 6.31 (1H, d, $J = 1.0$), 3.48 (1H, d, $J = 10.8$), 3.13 (1H, d, $J = 10.8$), 2.97 (1H, m), 1.32 (3H, s), 1.10 (3H, d, $J = 6.9$), 1.09 (3H, d, $J = 6.9$), 0.83 (3H, s); ^{13}C NMR (75 MHz) δ_C 187.9 (s), 187.8 (s), 152.6 (s), 150.6 (s), 142.6 (s), 131.7 (d), 71.4 (t), 44.5 (d), 38.2 (s), 37.6 (s), 35.8 (t), 34.5 (t), 26.1 (q), 25.4 (t), 21.2 (q), 21.2 (q), 20.4 (q), 18.2 (t), 17.6 (q), 16.9 (t); HRMS (EI) m/z 316.2045 $[M]^+$, calcd for $C_{20}H_{28}O_3$: 316.2038.

5.2 Biological assays (see ESI†)

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