Suppementary Data for

Cytotoxic, immunomodulatory, antimycotic, and antiviral activities of Semisynthetic 14-Hydroxyabietane Derivatives and Triptoquinone C-4 Epimers

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4.2.1 Compounds stock solutions.

Stock solutions of compounds were prepared in anhydrous dimethyl sulfoxide (DMSO, Sigma) and frozen at -70 ºC until required. The concentration of DMSO in biological assays was 0.05%. Cell controls with DMSO at 0.05% were used.

4.2.2 Antifungal assay.

The antifungal activity of compounds was evaluated following the Clinical and Laboratory Standards Institute M38-A protocol (CLSI) for filamentous fungi (National Committee for Clinical Laboratory Standards, Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi M38-A 2002).[1] *Aspergillus flavus* (ATCC 204304), *A. fumigatus* (ATCC 204305), *A. terreus* (CDC 317), and *A. niger* (ATCC 10124), cultured in potato dextrose agar (PDA), were used to evaluate antifungal activity. Briefly, seven serial dilutions of the compounds were dispensed into 96-well flat-bottomed microdilution plates in duplicate at final concentrations among 100 µg/mL and 2 µg/mL. Amphotericine B (Sigma Chemical Co., MO, USA) was used as positive control at a range of 16.0 - 0.031µg/mL. The plates were frozen at -70 ºC until required. The inoculum size for microdilution plates was 0.4–5 x 10^4 for filamentous fungi. In accordance with CLSI M38-A method, the MICs (corresponding to the 90% Minimun Inhibitory Concentrations, MIC_{90}) were determined after 48 h of incubation and defined as the lowest dilution that resulted in total inhibition of visible growth. MIC results were expressed as range and geometric mean (GM) of triplicates of each compound tested three times against each of the fungal species in different assays.

4.2.3 Antiviral assays.

Herpes simplex virus type 1 (HHV-1) was obtained from the Center for Disease Control (Atlanta, GA). The virus stock was prepared from HHV-1-infected HeLa cell cultures

(HeLa cell line ATCC CCL-2). The antiviral activity against HHV-1 has been carried out on HeLa and Vero cells using the end-point titration technique, according to the protocol reported by us,[2] which was used with a few modifications. Two-fold dilutions of the compounds and the viral suspension at one Tissue Culture Infectious Dose Fifty percent (1TCID50) were mixed and incubated for 0.5 h at 37 °C before they were added onto confluent monolayer cells and incubated again at 37 °C in a humidified 5% CO_2 atmosphere for 48 h. Acyclovir and Heparin sodium salt were used as positive controls.

4.2.4 Cells and cell culture.

The cell lines used were human *Cercopithecus aethiops* african green monkey kidney cells (Vero cell line ATCC CCL-81), human cervix epithelial adenocarcinoma cells (H1 HeLa cell line ATCC CRL-1958 and ATCC CCL-2) and acute T cell leukemia (Jurkat ATCC TIB-152). Vero and HeLa cells were grown in DMEM, supplemented with 10% and 5% of inactivated fetal bovine serum (FBS), respectively, 100 units/mL of penicillin, 100 µg/mL of streptomycin, 100 µg/mL of l-glutamine, 0.14% $NaHCO_3$, and 1% of each nonessential amino acids and minimum essential medium vitamin solution (choline chloride, D-Calcium pantothenate, Folic Acid, Nicotinamide, Pyridoxal hydrochloride, Riboflavin, Thiamine hydrochloride and i-Inositol). Jurkat cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 units/mL of penicillin, 100 µg/mL of streptomycin and 0.14% $NaHCO_3$. The cultures were maintained at 37 ºC in a humidified 5% CO_2 atmosphere.

Heparinised blood samples were obtained from healthy human volunteers; that is, without inflammatory, rheumatic, infectious or chronic diseases. Peripheral blood mononuclear cells were isolated by means of centrifugation on Histopaque for 30 min at 900 x *g*; the cells were then washed three times with sterile PBS, and they were suspended in RPMI-1640 medium supplemented with 10% inactivated FBS.

Finally, PBMC viability was determined by means of trypan blue staining, which had to be ≥ 98%.

4.2.5 Cell viability MTT assay.

Cell growth inhibition and/or cytotoxicity on Vero and HeLa, Jurkat cells was measured using a tetrazolium-dye (MTT) assay, according to the protocol previously described, with a few modifications.[3] Vero and HeLa cells were trypsinized and washed with phosphate buffered saline, and then plated at 1.35 x 10^4 and 1.25 x 10^4 cells per well, respectively, in a 96-well flat-bottomed plate; afterwards they were incubated for 24h. Moreover, Jurkat cells were centrifuged to remove medium, and they were again suspended in culture medium and plated at 3 x 10^4 cells per well in a 96-well round-bottomed plate. After that, each diluted compound was added to the appropriate wells, and the plates were incubated for further 48 h at 37 °C in a humidified incubator with 5% CO_2. Finally, spectrophotometric reading was carried out at 570 nm to determine the concentration for each compound that inhibited 50% of growth (IC_{50}).

4.2.6 CFSE Cell proliferation assay and CD4 and CD8 cells analysis.

Cell proliferation was measured using carboxyfluorescein diacetate succinimidyl (CFSE), following a previously described protocol with few modifications.[4] One million of Jurkat cells and PBMCs were suspended in Phosphate Buffer Saline (PBS) and incubated with CFSE (1 μg/mL) for 10 min at 37 °C in a humidified incubator with 5% CO_2, in the dark. After incubation, Jurkat cells and PBMCs were washed with 1 mL FBS and autologous serum, respectively. Then, the cells were suspended with medium supplemented with FBS for Jurkat cells or autologous serum for PBMCs. Labeling efficiency was verified by means of flow citometry, and it was above 90%.

Jurkat cells were plated at 5 x 10^4 in a 96-well round-bottomed plate and were treated with concentrations of active triptoquinones corresponding to IC_{50}, ½ IC_{50} and ¼ IC_{50}. After 48h of incubation, the cells were recollected and analyzed with a flow cytometer (Coulter Epics Facs XL).

PBMCs were plated at 1 x 10^5 per well in 96-well round-bottomed plate and treated with concentrations of active triptoquinones corresponding to IC_{50}, ½ IC_{50} and ¼ IC_{50} and stimulated with phytohaemaglutinin (PHA) (5 µg/mL) simultaneously. PBMCs were incubated for 72h to allow their differentiation; the cells were washed in PBS and stained with phycoerythrin-Cyanine 5 (PE-CyTM5) conjugated CD4 antibody (RPA-T4 BD, Biosciences Pharmingen, California-USA) or CD8 antibody (HIT8a, Biosciences Pharmingen, California-USA). Samples were analyzed with a flow cytometer (Coulter Epics Facs XL).

4.2.7 PBMC activation analysis.

The effect of active triptoquinone compounds on CD8 and CD4 cells activation was measured using CD69 antibody. Additionally, PBMCs were plated at 1 x 10^5 per well in a 96-well round-bottomed plate, and were treated with concentrations of active triptoquinones corresponding to IC_{50}, ½ IC_{50} and ¼ IC_{50}, one hour after or one hour before adding PHA (5 µg/mL). After 12h of incubation, PBMCs were recollected, washed in PBS and stained with PE conjugated CD69 antibody and PE-CyTM5 conjugated CD4 or CD8 antibodies. Samples were analyzed with a flow cytometer (Coulter Epics Facs XL).

4.2.8 Mitochondrial membrane potential and membrane integrity analysis

$DiOC_6(3)$ (3,3'-Dihexyloxacarbocyanine iodide) and propidium iodide (PI) were used to measure changes in mitochondrial membrane potential and membrane integrity

respectively, as previously described.[5] Briefly, Jurkat cells and PBMCs were plated at 5 x 10^4 and 16 x 10^4 per well respectively, in a 24-well flat-bottomed plate and treated at concentrations corresponding to IC50 and $_{1/2}IC_{50}$ for 15h and 24h. Then, cells were stained with $DiOC_6(3)$ (7 nM) and PI (1 µg/mL) for 30 min and washed in PBS. Samples were analyzed with a flow cytometer (Coulter Epics Facs XL).

4.2.9 Annexin V staining.

Phosphatidylserine exposure was determined using an Annexin V-PE (phycoerythrin) apoptosis kit (BD Biosciences Pharmingen, California-USA) according to the manufacturer's instructions. Briefly, Jurkat cells and PBMCs were plated at 5 x 10^4 and 16 x 10^4 per well respectively, in a 24-well flat-bottomed plate, and cells were then treated for 15h and 24h with a concentration corresponding to IC_{50} of each active triptoquinone. Then, the cells were collected by centrifugation, washed in PBS and incubated with PI (1 µg/mL) and AnnexinV-PE simultaneously, for 30 min to detect living, annexin V positive and necrotic cells. Samples were analyzed with a flow cytometer (Coulter Epics Facs XL).

4.2.10 Cell cycle distribution analysis.

Jurkat cells and PBMCs were plated at 5 x 10^4 and 16 x 10^4 per well respectively, in a 24-well flat-bottomed plate. Then, the cells were treated for 15h and 24h with concentrations of active triptoquinones corresponding to ½ IC_{50} and IC_{50}. After treatment, cells were collected and washed three times in PBS, and fixed in 70% ethanol for 15 min. Then cells were washed in PBS, and incubated with RNase (10 µg/mL) and PI (1 µg/mL) for 30 min at room temperature, following a protocol described elsewhere with few modifications.[6] Samples were analyzed with a flow cytometer (Coulter Epics Facs XL).

4.2.11 TUNEL assay

DNA punctual damage was measured using a MEBSTAIN kit (Medical & Biological laboratories Co, LTDA, Nagoya-Japon), according to the manufacturer's instructions. Jurkat cells and PBMCs were plated at 5 x 10^4 and 16 x 10^4 per well, respectively, in a 24-well flat-bottomed plate and then treated for 15h and 24h with concentrations corresponding to ½ IC_{50} and IC_{50} of each active triptoquinone. After treatment, the cells were collected by centrifugation, washed in PBS, fixed in 2% paraformaldehyde and incubated with TdT buffer (terminal deoxynucleotidyl transferase), TdT solution (TdT buffer, fluorescein-2´-Deoxyuridine, 5´-Triphosphate (FITC-dUTP), TdT) and TB solution for 10, 60 and 15 min. Finally, the resulting cells were incubated with PI for 20 min. Samples were analyzed with a flow cytometer (Coulter Epics Facs XL).

4.2.12 Effect on differentiation of mononuclear phagocytes.

Isolated PBMCs were stained with anti-CD14-CyChorme (Becton Dickinson-Pharmingen, San Diego,CA). PBMCs containing 3 x 10^5 $CD14^+$ cells were plated in 48-well plates (Corning Incorporated Life Science. Lowell, MA) using 1 ml of RPMI-1640 plus 0.5% pooled human serum (PHS), during 4 h at 37°C. Then, wells were extensively washed with pre-warmed PBS plus 0.5% PHS to remove non-adherent cells. Adherent cells were cultured in 1 ml of RPMI-1640 supplemented with 10% PHS and in the presence or absence of the compounds for 120 h to allow differentiation into monocyte derived macrophages (MDM). The efficiency of purification was more than 90% (HLA class II^+ $CD14^+$). Adherent cells were enumerated by scrapping or lysing to count cells and nuclei, respectively, as described before.[7]

The differentiated phagocytes (2.5 x 10^5) were stained with anti-HLA-DR PE (Becton Dickinson-Pharmingen, San Diego,CA) for 30 min at room temperature. Also,

differentiated phagocytes were stained with 250 ng/ml fluorescein diacetate (FDA) (Invitrogen, Eugene, OR) at 37 °C for 60 min. Samples were analyzed with a flow cytometer (Coulter Epics Facs XL).

4.2.13 Cytokine production of monocytes.

After 120 h of differentiation, the culture medium of differentiated cells was replaced and cells were treated with 100 ng/ml of lipopolysaccharide (LPS from Escherichia coli, Sigma Aldrich) for 24 h. Then, TNF-α, IL-1β and IL-10 levels were determined in supernatants through Cytometric Bead Array (CBA, Becton Dickinson-Pharmingen).

4.3 Statistical analysis.

IC_{50} was obtained from dose-effect curves through linear regression methods and values were presented as the mean ± SD (standard deviation) of least four dilutions by quadruplicate. To define which compounds were more selective to cancerous cells than to non-cancerous cells, selectivity index (SI) was calculated, defined as Vero IC_{50} over Jurkat or HeLa IC_{50}. A compound with a SI > 5 was considered to be selective.

The differences between groups were assessed using ANOVA, followed by Tuckey post-hoc analysis. Data were presented as mean ± ANOVA combined standard error. Values of $p < 0.05$ were considered to be significant as compared to the untreated control. All assays were carried out in triplicate, except the Annexin V assay, because this was confirmatory.

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FIGURES 2-10

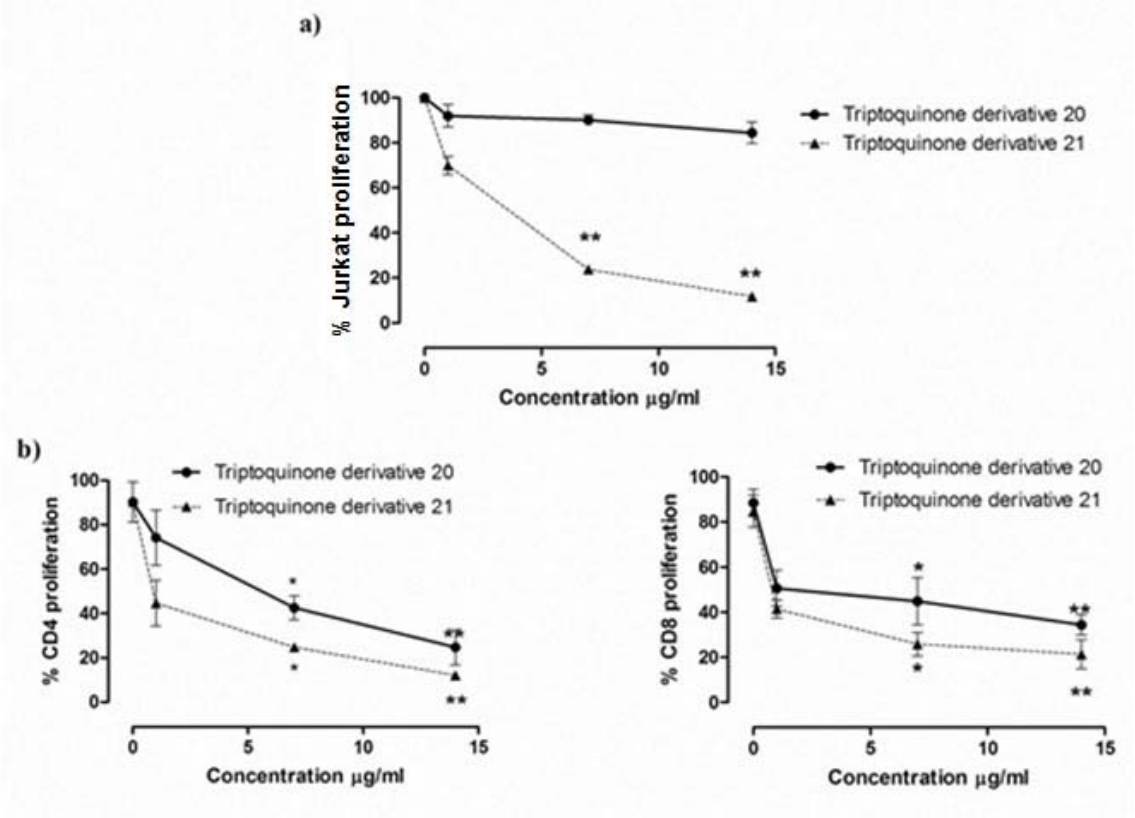

Fig. 2. **Jurkat and PBMC cell proliferation assay** Jurkat and PBMC were treated with different concentrations of triptoquinone epimers for 48h and 72h, respectively, and then cell proliferation was measured using CFSE dye. Concentration of 0 correspond to control no treated. Data were expressed as mean ± ANOVA combined standard error of three independent assays. ** $p<0.01$, *$p<0.05$ compared to not treated control. a) % proliferation inhibition of Jurkat cells was calculated comparing mean fluorescence intensity (MFI) of treated cells respect to control's MFI (100%). b) % proliferation of CD4 and CD8 cells was calculated comparing MFI of treated cells respect to control (100%).

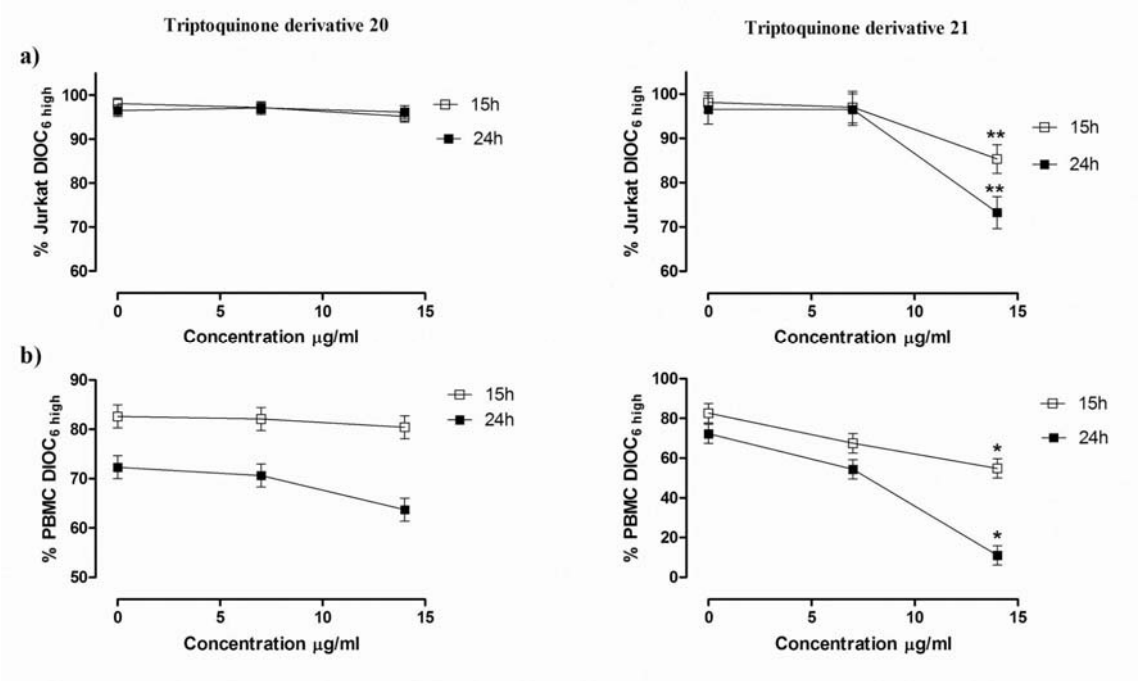

Fig. 3. **Jurkat and PBMC mitochondrial membrane potencial and plasmatic membrane integrity.** The cells were treated with triptoquinone epimers at the concentrations of 7.0 and 14.0 µg/mL for 15 h and 24 h, and then mitochondrial potential was measured using DiOC6 dye. Percentage of Jurkat Cells (a) and PBMC (b) with high potential after 15 h and 24 h of treatment with triptoquinone epimers at concentrations of 7.0 and 14.0 µg/mL. Concentration of 0 correspond to control no treated. Data were expressed as mean ± ANOVA combined standard error of three independent assays.d ** $p<0.01$, * $p<0.05$ compared to not treated control.

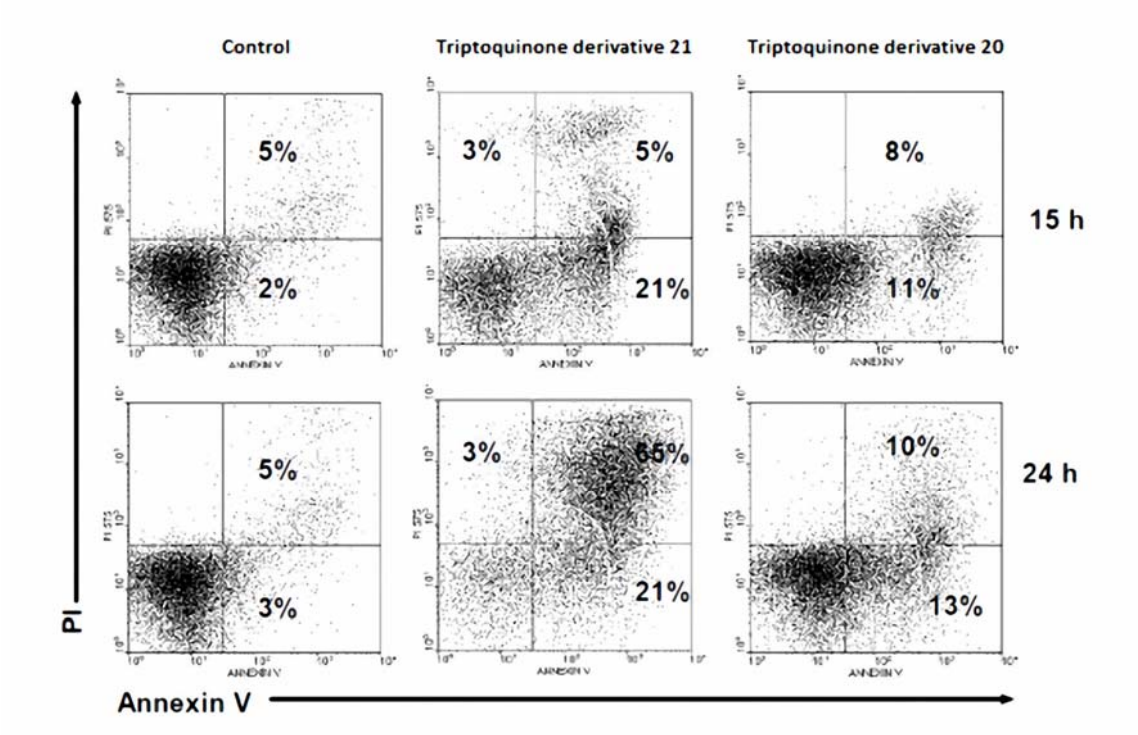

Fig. 4. **Jurkat phosphatidylserine exposure.** Jurkat cells were treated with triptoquinone epimers at a concentration of 14.0 μg/mL for 15 h and 24 h, and then phosphatidylserine exposure and membrane integrity was measured. The viable cells, Annexin V positive cells, Annexin V and PI positive cells and necrotic cells are present in the lower left, lower right, upper right and upper left quadrant, respectively.

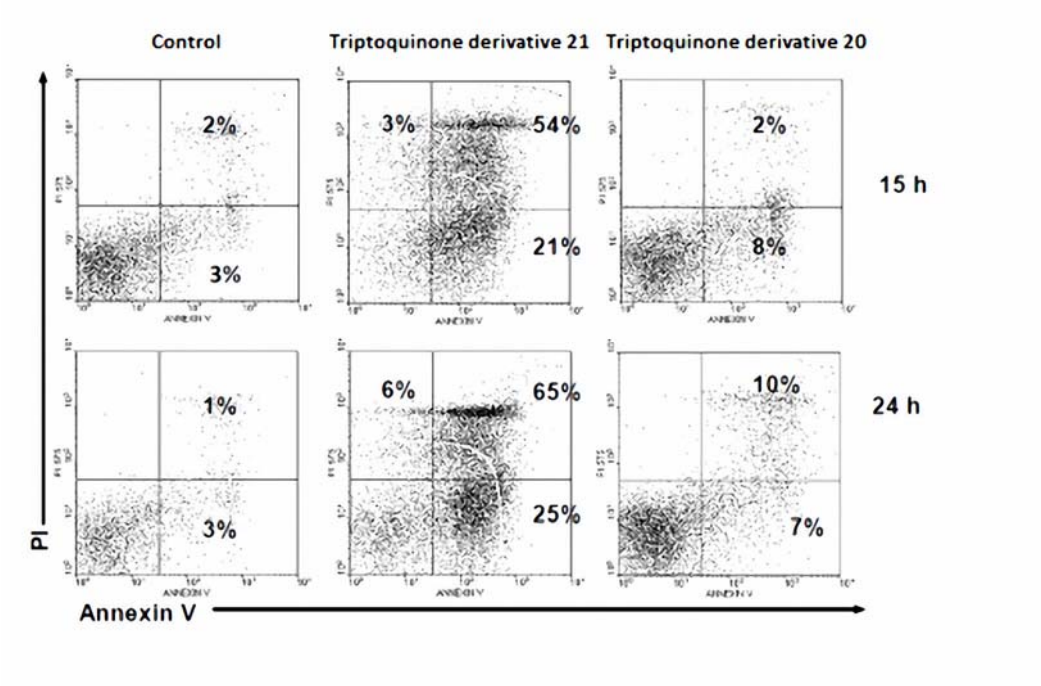

Fig. 5. **PBMC phosphatidylserine exposure.** PBMC were treated with triptoquinone epimers at a concentration of 14.0 µg/mL for 15 h and 24 h, and then phosphatidylserine exposure and membrane integrity was measured. The viable cells, Annexin V positive cells, Annexin V and PI positive cells and necrotic cells are present in the lower left, lower right, upper right and upper left quadrant, respectively.

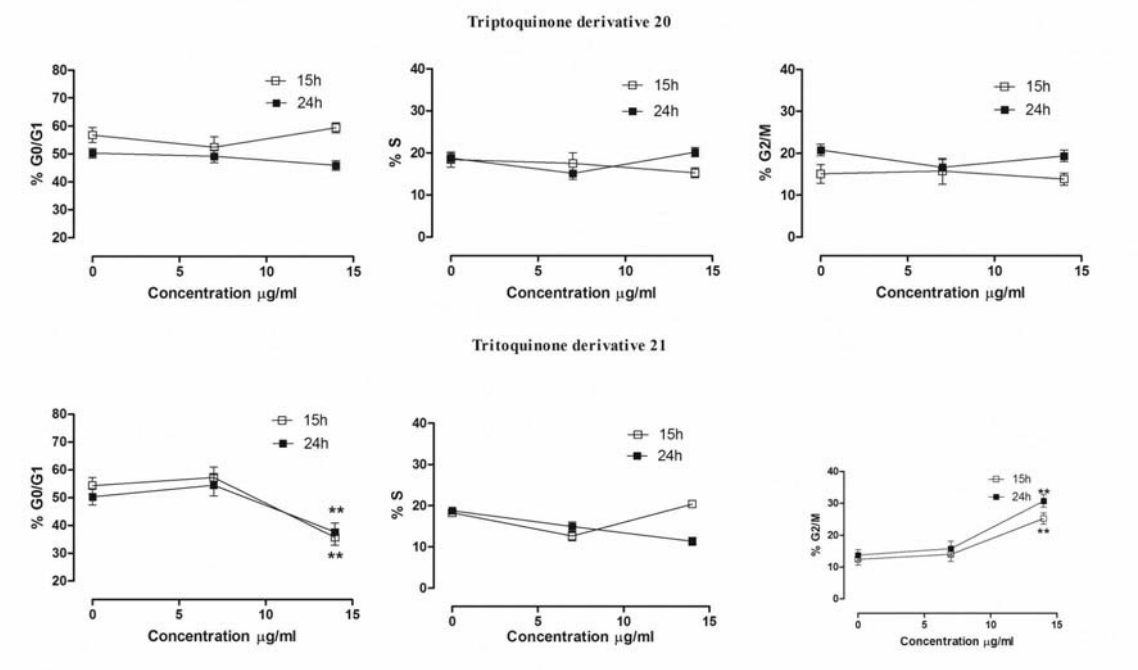

Fig. 6. **Jurkat cell cycle distribution.** Jurkat cells were treated with triptoquinone epimers at the concentrations of 7.0 and 14.0 µg/mL for 15 h and 24 h, and then cell cycle distribution was analyzed by flow cytometry. Concentration of 0 correspond to control no treated. Data were expressed as mean ± ANOVA combined standard error of three independent assays. ** $p<0.01$, compared to control not treated.

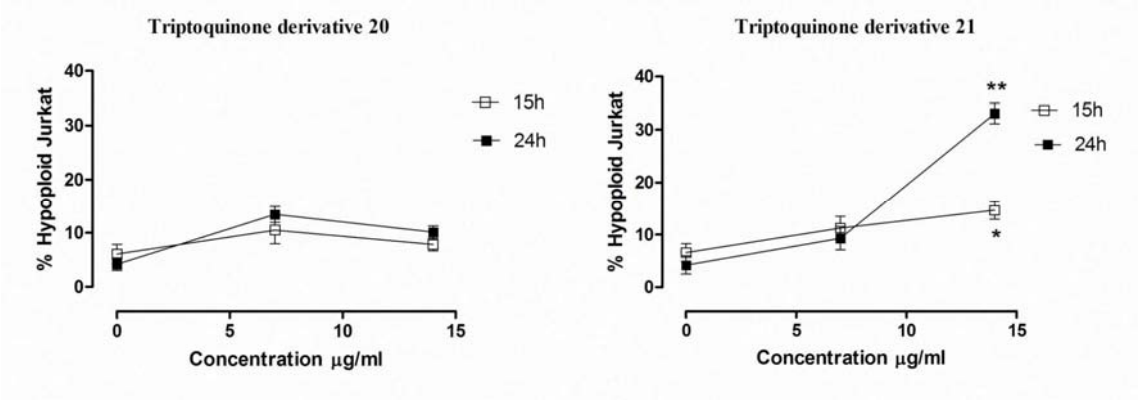

Fig. 7. **Jurkat hypopoidy measurement.** The cells were treated with triptoquinone epimers at the concentrations of 7.0 and 14.0 µg/mL for 15 h and 24 h, and then hypoploidy was measured using PI. Concentration of 0 correspond to control no treated. Data were expressed as mean ± ANOVA combined standard error of three independent assays. ** $p<0.01$, *$p<0.05$ compared to control not treated.

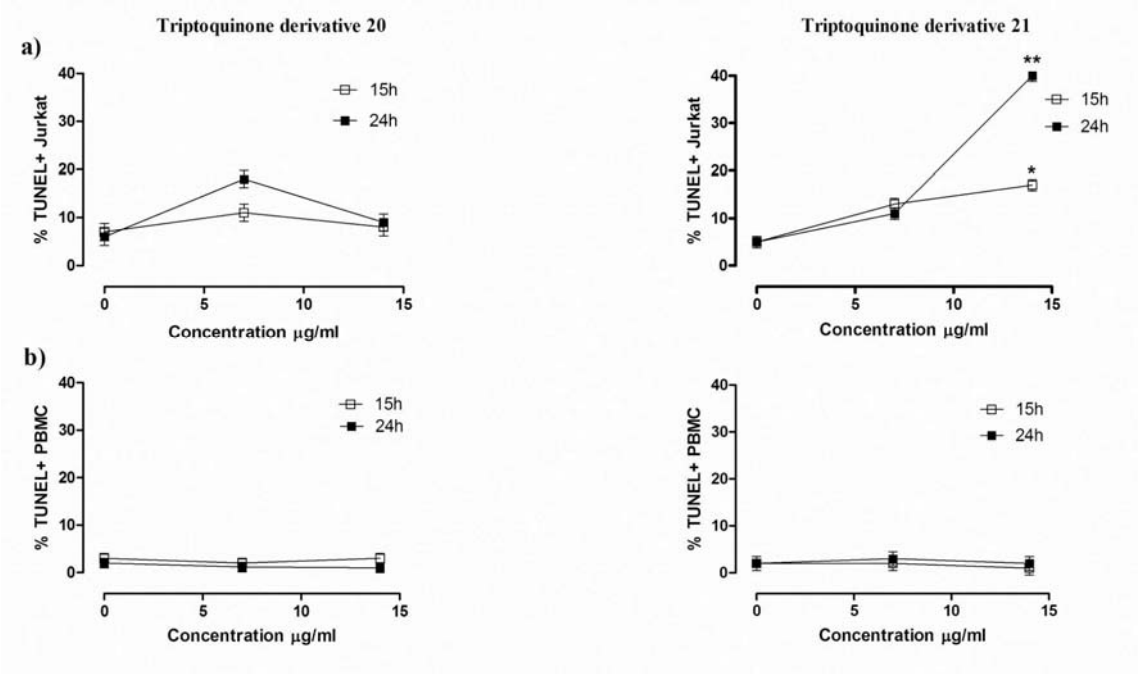

Fig. 8. **Jurkat and PBMC DNA damage-TUNEL assay.** The cells were treated with triptoquinone epimers at the concentrations of 7.0 and 14.0 µg/mL for 15 h and 24 h, and then DNA damage was measured by TUNEL assay. Percentage of Jurkat cells (a) and PBMC (b) TUNEL+ respect to control. Concentration of 0 correspond to control no treated. Data were expressed as mean ± ANOVA combined standard error of three independent assays. ** $p<0.01$, *$p<0.05$ compared to control not treated.

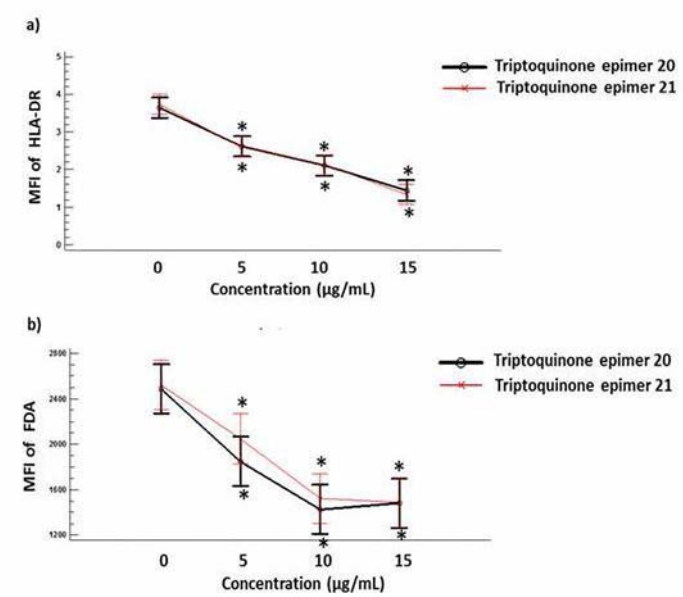

Fig.9. **PBMC HLA-DR expression.** Cells were incubated with active triptoquinones at concentrations of 5, 10 and 15 µg/mL for 120 h, and then HLA-DR expression (a) and effect on monocyte differentiation (b) were measured. Data were expressed as mean ± ANOVA combined standard error of three independent assays.

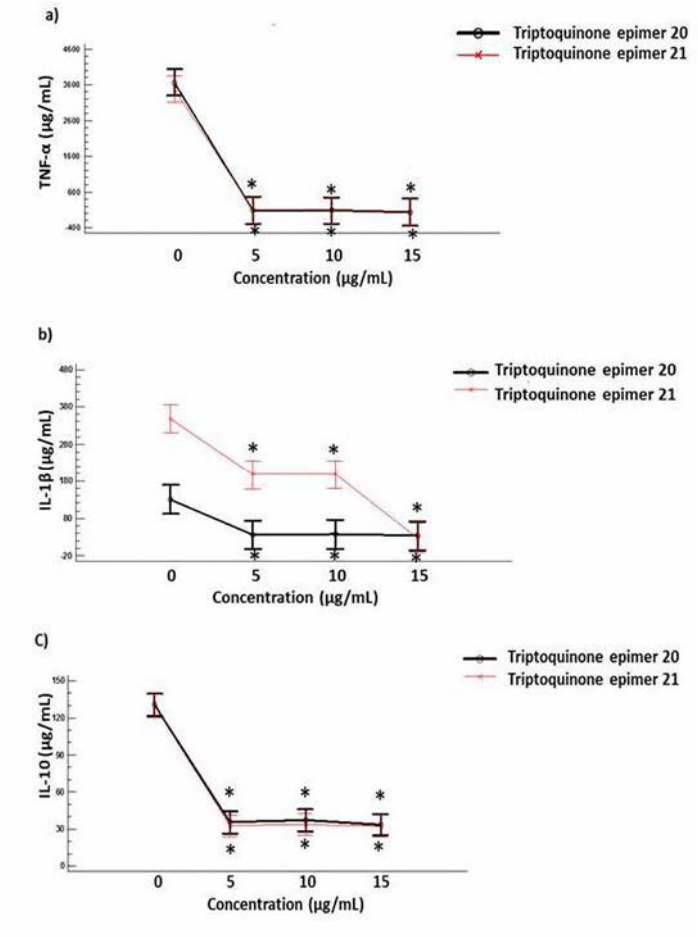

Fig.10. **PBMC cytokine production.** Cells were incubated with active triptoquinones at concentrations of 5, 10 and 15 µg/mL for 120 h and then cells were treated with 100 ng/ml of LPS for 24 h and TNF-α, IL-1β and IL-10 levels were determined in supernatants by Cytometric Bead Array. Data were expressed as mean ± ANOVA combined standard error of three independent assays. *$p<0.05$ compared to control not treated.