

## Diterpenes from *Xylopi*a *langsdo*rffiana Inhibit Cell Growth and Induce Differentiation in Human Leukemia Cells

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Two new diterpenes were isolated from stems and leaves of *Xylopi*a *langsdo*rffiana, *ent*-atisane-7 $\alpha$ ,16 $\alpha$ -diol (xyloidiol) and *ent*-7 $\alpha$ -acetoxytrachyloban-18-oic acid (trachylobane), along with the known 8(17),12*E*,14-labdatrien-18-oic acid (labdane). We investigated their antitumour effects on HL60, U937 and K562 human leukemia cell lines. We found that xyloidiol was the most potent diterpene in inhibiting cell proliferation of HL60, U937 and K562 cells, with mean IC<sub>50</sub> values of 90, 80 and 50  $\mu$ M, respectively. Based on the nitroblue tetrazolium (NBT) reduction assay, all the diterpenes were found to induce terminal differentiation in HL60 and K562 cells, with xyloidiol being the most effective. NBT reduction was increased by almost 120% after 12 h exposure of HL60 cells to xyloidiol at a concentration lower than the IC<sub>50</sub> (50  $\mu$ M). Thus, xyloidiol inhibited human leukemia cell growth *in vitro* partly by inducing cell differentiation, and merits further studies to examine its mechanism of action as a potential antitumoural agent.

*Key words:* *Xylopi*a *langsdo*rffiana, Diterpenes, Cytotoxicity

### Introduction

Hematopoietic cells undergo a genetically regulated program of maturation in which pluripotential stem cells gradually acquire the lineage specificity and functional characteristics of their more differentiated counterparts. This phenomenon is regularly accompanied by a reciprocal reduction in self-renewal capacity. Thus, the failure of leukemic cells to undergo differentiation represents a prototypical example of dysregulated differentiation as a fundamental hallmark of neoplastic transformation (Leszczyniecka *et al.*, 2001). A potentially less toxic form of cancer therapy involves the use of agents, alone or in combination, that modify the differentiation state and growth of cancer cells (Leszczyniecka *et al.*, 2001; Jiang *et al.*, 1994). Thus, cells exposed to chemical or biological agents are driven toward a differentiation pathway that results in end-stage adult cells with no replicative capacity, which triggers apoptosis (Anazetti *et al.*, 2003). Human cell lines derived from myeloid leukemias have been widely used

as models to study the molecular control of hematopoietic cell proliferation and differentiation.

For many centuries, plants have been a main source for drug development. From 1941 to 2002, over 50% of all the drugs, or new drug entities, available for cancer treatment were derived from natural resources (Newman *et al.*, 2003). The genus *Xylopi*a (Annonaceae) comprises about 160 species (Maas *et al.*, 2001). Various species of this genus are used in folk medicine against rheumatism (Agelita *et al.*, 2001) and as antimicrobial agents (Correa, 1984). Kaurane-, labdane- and trachylobane-type diterpenes are encountered in this genus (Faulkner *et al.*, 1985; Andrade *et al.*, 2004; Morais and Roque, 1988). Atisane-type diterpenes are secondary metabolites, quite rare in nature, and their bioactivities are poorly understood. Atisane diterpenes have been recorded only in *Xylopi*a *aromatica* among the species of the family Annonaceae (Morais and Roque, 1988). However, other atisane-type diterpenes isolated from different species show anti-HIV (Sun *et al.*, 2003) and insecticidal activities and are cytotoxic

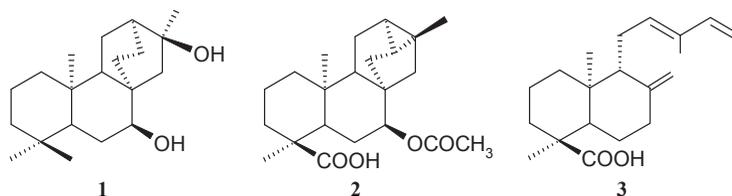


Fig. 1. Chemical structure of xylodiol (**1**), trachylobane (**2**), and labdane (**3**).

to P388 mouse leukemia cells (Perry *et al.*, 2001). Various terpenoids are natural compounds attractive as therapeutic agents for the treatment of cancer (Kondoh *et al.*, 2004). Labdane-type diterpenes have been reported to have a broad spectrum of biological activities, including cytotoxic, cytostatic and antineoplastic (Dimas *et al.*, 2001; Li *et al.*, 2005). In addition, trachylobane diterpenes have already shown cytotoxic activity against leukemia cells by apoptotic pathways (Graikou *et al.*, 2004; Block *et al.*, 2004, 2005).

*Xylopi* *langsdorffiana* St.-Hil. & Tul. is a tree, 5–7 m in height and popularly known in Northeast Brazil as “pimenteira da terra” (Correa, 1984). Recently, our group reported the isolation and characterization of two new diterpenes, *ent*-*atisan*-7 $\alpha$ ,16 $\alpha$ -diol (xylodiol) (**1**) (Tavares *et al.*, 2007) and *ent*-7 $\alpha$ -acetoxytrachyloban-18-oic acid (trachylobane) (**2**) (Tavares *et al.*, 2006), together with the known 8(17),12*E*,14-labdatrien-18-oic acid (labdane) (**3**) (Tavares *et al.*, 2007) from *X. langsdorffiana* stems and leaves (Fig. 1). Vasorelaxant and hypotensive activities were reported recently for labdane (Oliveira *et al.*, 2006). Our previous data showed that all of them exhibited weak cytotoxic effects on V79 fibroblast and rat hepatocytes (Tavares *et al.*, 2006, 2007).

As a very valuable source for novel chemotherapeutic agents, natural plant compounds exhibit effective antitumour activities with a wide range of mechanisms. In the present study, we used human leukemia cell lines, including HL60, U937 and K562, to investigate the anticancer effects of diterpenes from *Xylopi* *langsdorffiana* in terms of induction of differentiation.

## Experimental

### Chemicals and cell lines

The diterpenes xylodiol (atisane), trachylobane, and labdane were obtained from *Xylopi*

*langsdorffiana* stems and leaves as previously described (Tavares *et al.*, 2006, 2007).

The identification of labdane was carried out by the analysis of  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectral data compared with those published in the literature, and the structural elucidation of xylodiol and trachylobane was based on various spectral data (UV, IR,  $[\alpha]_D^{25}$ , MS,  $^1\text{H}$  and  $^{13}\text{C}$  NMR, 1D and 2D). The physicochemical properties of the diterpenes were in perfect accordance with data reported for the structures of the compounds depicted in Fig. 1 (Tavares *et al.*, 2006, 2007).

The leukemia cells (HL60, U937, and K562) were grown in suspension at 37 °C in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml of penicillin and 100  $\mu\text{g}/\text{ml}$  of streptomycin in a humidified atmosphere containing 5%  $\text{CO}_2$  in air. To assess viability, the cells ( $3 \cdot 10^5/\text{ml}$ ) were seeded in 96-well plates and incubated with different concentrations of diterpenes for 72 h. Cell viability was determined by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) and phosphatase activity. For the nitroblue tetrazolium (NBT) reduction assay, the cells ( $3 \cdot 10^5/\text{ml}$ ) were cultured in flasks and incubated with each diterpene at concentrations close to the 50% inhibitory concentration ( $\text{IC}_{50}$ ), for 12, 24, 48 and 72 h. The compounds were first dissolved in dimethyl sulfoxide (DMSO) and then diluted in supplemented medium. The final content of DMSO in the test medium and controls was 0.1%.

### *In vitro* cell-growth inhibition assays

The tetrazolium reduction assay was performed as described by Denizot and Lang (1986). Briefly, 0.1 ml of serum-free medium containing MTT (1 mg/ml) was added to each well. After incubation for 4 h, the supernatant was removed and the blue formazan product obtained was dissolved in 1 ml of ethanol with stirring for 15 min on a

microplate shaker after which the absorbance at 570 nm was read.

After incubation for 72 h with the diterpenes, the phosphatase activity of HL60, U937, and K562 cells was assayed as described by Anazetti *et al.* (2003). The culture medium was carefully removed from the wells and *p*-nitrophenyl phosphate dissolved in 1 M acetate buffer, pH 5.5, at a final concentration of 75 mM was added. After 30 min incubation on a microplate shaker at room temperature, the reaction was stopped by adding NaOH. The resulting absorbance was read at 405 nm.

The cell viability experiments were performed at least three times with six replicates each time. The IC<sub>50</sub> values were calculated after expressing the results as percentage of the controls and were determined graphically from the concentration-response curves.

#### NBT reducing activity

Cells ( $3 \cdot 10^5$ /ml) were cultured with each diterpene at concentrations close to the IC<sub>50</sub> value in RPMI-1640 medium containing 10% FBS for 12, 24, 48 and 72 h, after which the NBT reducing activity was determined by the method of Kohroki *et al.* (1998), with slight modifications. Briefly, the cells ( $2 \cdot 10^6$ /ml) were harvested by centrifugation and resuspended in 1 ml of RPMI-1640

medium containing 20% FBS. After the addition of 500  $\mu$ l of a 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (200  $\mu$ g/ml)-NBT (1 mg/ml) solution, the cells were incubated at 37 °C in 5% CO<sub>2</sub> for 25–30 min. After centrifugation, 600  $\mu$ l of ethanol were added to the cell pellet to solubilize the formazan deposit. The amount of formazan formed was determined by reading the absorbance at 560 nm. Statistical analyses were performed using one-way ANOVA or Student's *t*-test.

#### Results and Discussion

All the diterpenes exhibited significant concentration-dependent inhibitory effects on the proliferation of HL60 cells. Xylodiol was the most growth-inhibitory diterpene in HL60 cells as shown in Fig. 2A. A similar inhibitory effect was found for xylodiol using the MTT viability assay (IC<sub>50</sub> = 90  $\mu$ M) and phosphatase activity (IC<sub>50</sub> = 75  $\mu$ M). Fig. 2B shows that labdane was less toxic to HL60 cells than were xylodiol and trachylobane. Labdane showed similar growth inhibition evaluated by MTT reduction and phosphatase activity (IC<sub>50</sub> = 300  $\mu$ M and 280  $\mu$ M, respectively). IC<sub>50</sub> values obtained for trachylobane were 127  $\mu$ M and 96  $\mu$ M evaluated by the two assays, respectively, and therefore, trachylobane had an intermediate cytotoxicity (Fig. 2C).

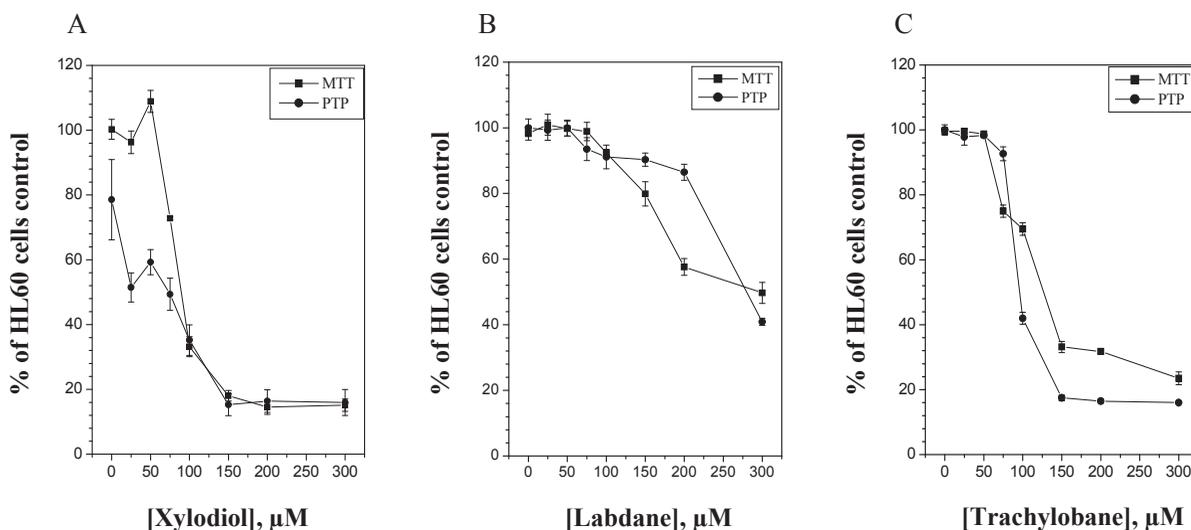


Fig. 2. Viability of cultured HL60 cells after treatment with (A) xylodiol, (B) labdane, and (C) trachylobane for 72 h (MTT reduction and phosphatase activity).

As shown in Fig. 3, all the diterpenes inhibited the growth of U937 cells in a concentration-dependent manner. Proliferation of U937 cells was most significantly reduced by xylodiol, whose  $IC_{50}$  values were  $76 \mu\text{M}$  and  $87 \mu\text{M}$  for MTT reduction and phosphatase activity, respectively (Fig. 3A). Fig. 3B shows that labdane was the weakest growth-inhibitory diterpene in U937 cells evaluated by MTT reduction ( $IC_{50} = 252 \mu\text{M}$ ) and phosphatase activity ( $IC_{50} = 100 \mu\text{M}$ ).  $IC_{50}$  values for

trachylobane were  $156 \mu\text{M}$  and  $166 \mu\text{M}$  determined by the two assays, respectively (Fig. 3C).

Upon treatment of K562 cells with xylodiol and trachylobane, ranging from 0 to  $300 \mu\text{M}$ , it was found that cell growth was inhibited by these diterpenes in a concentration-dependent manner. The  $IC_{50}$  values for xylodiol were  $40 \mu\text{M}$  and  $55 \mu\text{M}$  for MTT reduction and phosphatase activity assays, respectively (Fig. 4A). Trachylobane showed intermediate growth inhibition when compared

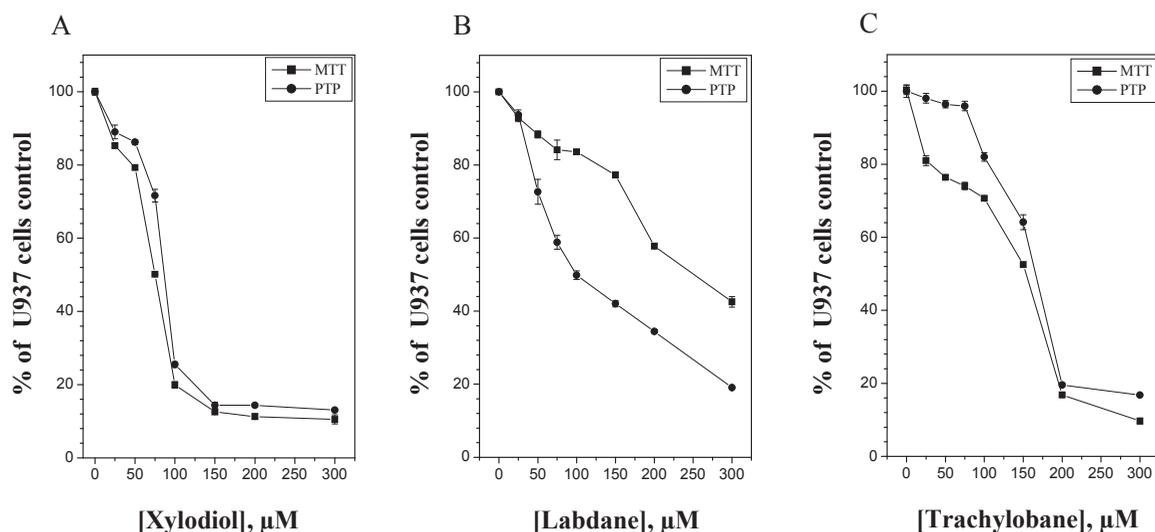


Fig. 3. Viability of cultured U937 cells after treatment with (A) xylodiol, (B) labdane, and (C) trachylobane for 72 h (MTT reduction and phosphatase activity).

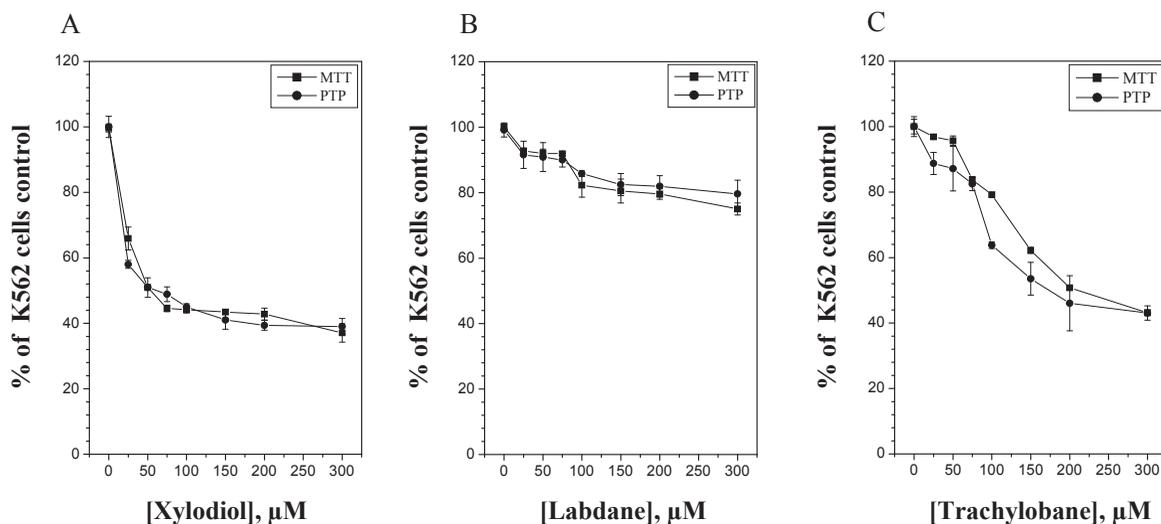


Fig. 4. Viability of cultured K562 cells after treatment with (A) xylodiol, (B) labdane, and (C) trachylobane for 72 h (MTT reduction and phosphatase activity).

with xyloidiol and labdane (Fig. 4C). The  $IC_{50}$  values were  $207 \mu M$  and  $172 \mu M$  for MTT reduction and phosphatase activity, respectively. These results indicate that xyloidiol and trachylobane have antiproliferative activity in K562 cells. However, labdane had no significant effect on the growth of K562 cells (Fig. 4B).

The *in vitro* assays using mammalian cell cultures for the evaluation of cytotoxic effects of xenobiotics are based on the assessment of different cellular endpoints which provides information on the susceptibility of cellular organelles and compartments. Reduction of MTT assesses the mitochondrial function through the activity of succinate dehydrogenase. Phosphatases participate in

various cellular processes and the activity of these enzymes can be used to determine the cell viability (Anazetti *et al.*, 2003). Using these viability tests, xyloidiol was the most toxic diterpene to human leukemia cells. Trachylobane showed intermediate cytotoxicity and labdane was the weakest active against leukemia cells.

The 'differentiation therapy' is an approach based on the assumption that many neoplastic cell types exhibit reversible defects in differentiation, which, upon appropriate treatment, results in tumour reprogramming and a concomitant loss in proliferative capacity and induction of terminal differentiation and apoptosis (programmed cell death) (Jiang *et al.*, 1994; Leszczyniecka *et al.*, 2001).

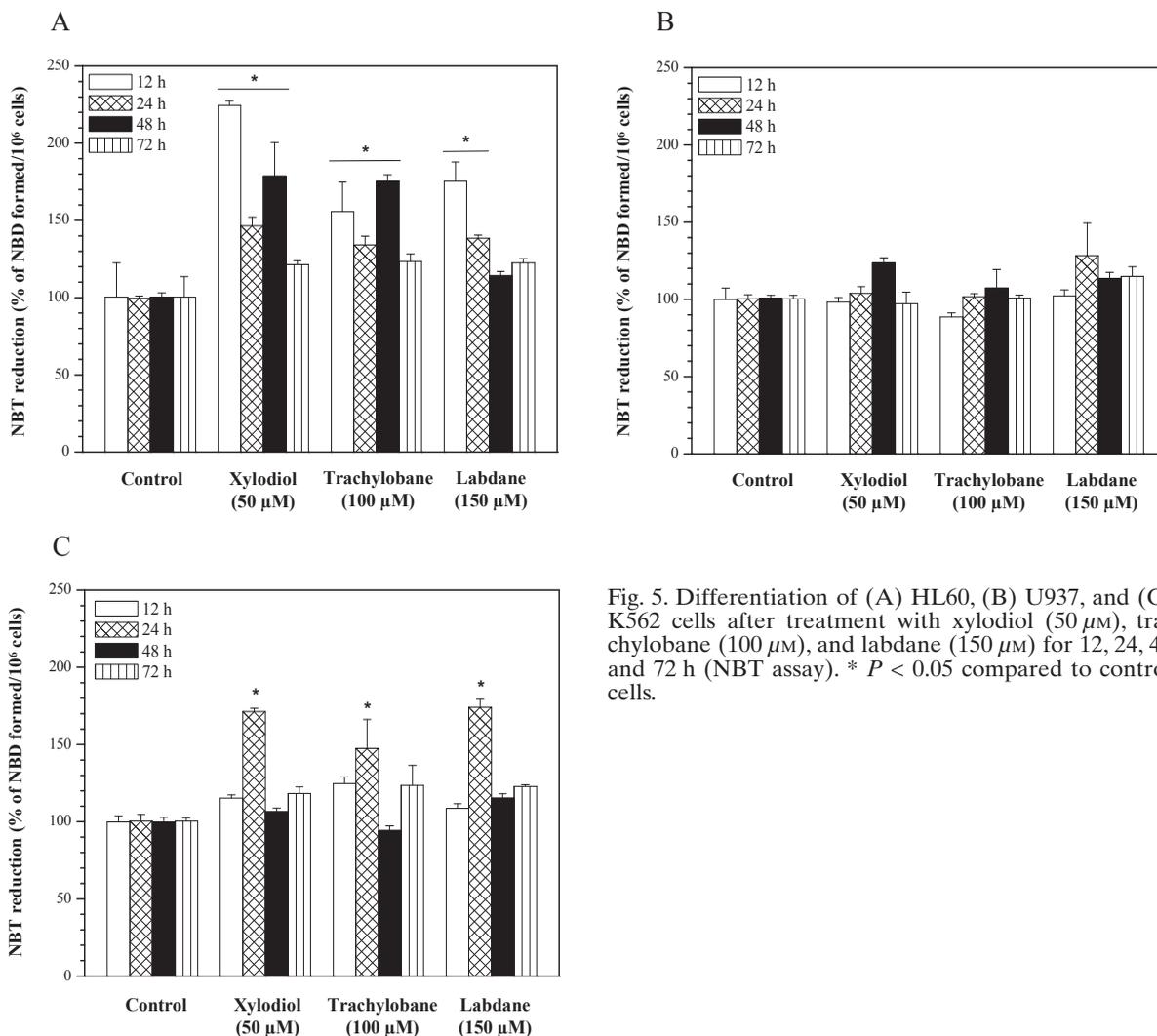


Fig. 5. Differentiation of (A) HL60, (B) U937, and (C) K562 cells after treatment with xyloidiol ( $50 \mu M$ ), trachylobane ( $100 \mu M$ ), and labdane ( $150 \mu M$ ) for 12, 24, 48 and 72 h (NBT assay). \*  $P < 0.05$  compared to control cells.

The capability of reducing NBT is the first parameter to investigate functional differentiation. To explore the ability of diterpenes to induce differentiation in leukemia cells, the NBT reduction activity was assessed in cells treated with xyloidiol (50  $\mu\text{M}$ ), labdane (150  $\mu\text{M}$ ), and trachylobane (100  $\mu\text{M}$ ) for 12, 24, 48 and 72 h.

As shown in Fig. 5A, xyloidiol strongly enhanced the degree of cell differentiation at a concentration lower than the  $\text{IC}_{50}$  of 75  $\mu\text{M}$  within 12 h of treatment. NBT reduction was increased by almost 120% after 12 h of exposure to xyloidiol. There was a lower NBT reducing activity in HL60 cells treated with trachylobane, approx. 55% after 12 h and 75% after 48 h. According to Fig. 5A, labdane increased the NBD (nitroblue diformazan) formation by about 75% after 12 h of treatment. These results showed that all the diterpenes studied induced the differentiation of HL60 cells, with xyloidiol being the most effective. All three diterpenes induced no significant U937 cells differentiation according to our experimental conditions (Fig. 5B). In contrast to the control cells, results from the NBT reduction assay showed that the number of differentiated K562 cells increased significantly after treatment with xyloidiol and labdane by about 70% after 24 h.

At 100  $\mu\text{M}$ , trachylobane increased the NBD production by approx. 47% after 24 h of treatment (Fig. 5C). Thus, the diterpenes induced K562 cells differentiation, with xyloidiol and labdane being more effective than trachylobane.

The distinct abilities in inducing the differentiation of HL60, U937, and K562 cells may be due to differential gene expression in different cell types. The mechanism by which diterpenes induce differentiation in HL60 and K562 cells still remains unknown. Considering that the NBT assay is rather non-specific, since both monocytes and granulocytes can stain positive, future studies should include a more specific determination of the cell types present in the cultures after treatment.

Thus, the present study showed that xyloidiol has notable antileukemic effects by inducing cell differentiation. These data suggest that xyloidiol, as a natural substance with growth inhibition and differentiation effects on leukemia cells, merits further studies to examine its mechanism of action as antitumoural agent.

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