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Article

Lupeol and its esters: NMR, powder XRD data and *in vitro* evaluation of cancer cell growth

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The triterpene lupeol (**1**) and some of its esters are secondary metabolites produced by species of Celastraceae family, which have being associated with cytotoxic activity. We report herein the isolation of **1**, the semi-synthesis of eight lupeol esters and the evaluation of their *in vitro* activity against nine strains of cancer cells. The reaction of carboxylic acids with **1** and DIC/DMAP was used to obtain lupeol stearate **(2)**, lupeol palmitate **(3)** lupeol miristate **(4)**, and the new esters lupeol laurate **(5)**, lupeol caprate **(6)**, lupeol caprilate **(7)**, lupeol caproate (**8**) and lupeol 3',4'-dimethoxybenzoate (**9**), with high yields. Compounds **1**-**9** were identified using FT-IR, ^{1}H, ^{13}C-NMR, CHN analysis and XRD data and were tested *in vitro* for proliferation of human cancer cell activity. In these assays, lupeol was inactive (GI_{50}> 250μg/mL) while lupeol esters **2** -**4** and **7** - **9** showed a cytostatic effect. The XRD method was a suitable tool to determine the structure of lupeol and its esters in solid state. Compound **3** showed a selective growth inhibition effect on erythromyeloblastoid leukemia (K-562) cells in a concentration-dependent way. Lupeol esters **4** and **9** showed a selective cytostatic effect with low GI_{50} values representing promising prototypes for the development of new anticancer drugs.

Keywords: Lupeol/*in vitro* evaluation. Lupeol ester. K-562 cells. XRD method. Antiproliferative effect.

INTRODUCTION

Despite the efforts to develop new strategies of cancer prevention and therapy (Galmarini, Galmarini, Galmarini, 2012), cancers still represent a worldwide problem of public health. According to the Word Health Organization (Ferlay *et al.*, 2012), around 14 million of the new cancer cases occurred in 2012 (57% of this total in less developed regions). Among the strategies to treat cancer, cancer chemotherapeutic agents represent crucial tools basically aiming to eliminate or at least inhibit tumor cell growth (Galmarini, Galmarini, Galmarini, 2012; Chabner, Roberts, 2005). Considering all antitumor chemotherapeutic arsenal approved between 1940s and 2014, 49% (85 chemical entities) were natural products *per si* or directly derived from them (Newman, Cragg, 2016).

Among natural products, triterpenoids have been considered a promising class for cancer chemoprevention and chemotherapy (Salminen *et al.,* 2008; Lachance *et al.*, 2012; Gali-Muhtasib *et al.*, 2015), and they have been highlighted as antineoplastic agents (DallaVechia, Gnoatto, Gosmann, 2009; Laszczyk, 2009; Siddique, Saleem, 2011; Sultana, 2011).

Triterpenes inhibit tumor growth, cellular cycle progression, and induce the apoptosis of tumor cells both *in vitro* and *in vivo* tests besides presenting anti-

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inflammatory, antioxidant and antiangiogenic effects (Laszczyk, 2009; Siddique, Saleem, 2011). Lupeol (**1**, Figure 1), a pentacyclic triterpene, occurs in many medicinal plants (Laszczyk, 2009), such as in leaves of *Maytenus salicifolia* Reissek (Celastraceae) (Núñez *et al.*, 2005). This compound has displayed anti-inflammatory property (Salminen *et al.*, 2008; Saleem, 2009; Shahlaei *et al.*, 2013), protective effect during low-density lipoprotein (LDL) oxidation (Geetha, Varalakshmiu, Latha, 1998; Andrikopoulos *et al.*, 2003), and anticancer activity against different cell lines [melanoma (G361, 451Lu and WM35), T-lymphoblastic leukemia (CEM), breast carcinoma (MCF-7 and MDA-MB-231), lung carcinoma (A-549), multiple myeloma (RPMI 8226) and cervical carcinoma (HeLa)] (Saleem, 2009; Saleem *et al.*; 2008; Gallo, Sarachine, 2009).

Some natural lupeol esters also present promising biological effects such as antimalarial (Fotie *et al.*, 2006) and acetylcholinesterase inhibitory activities (Gurovic *et al.*, 2010). Based on these promising activities, some lupeol esters have been synthetized and evaluated for different activities (Li *et al.*, 2013; Lachance *et al.*, 2012; Reddy *et al.*, 2009; Sudhahar, Kumar, Varalaksmi, 2006a). For example, lupeol linoleate has been described as effective to reduce hypercholesterolemia (Sudhahar, Kumar, Varalaksmi, 2006a; Sudhahar, Kumar, Varalaksmi, 2006b; Sudhahar *et al.*, 2007a) and also as a protective agent in different oxidative stress conditions (Sudhahar, Kumar, Varalaksmi, 2006a; Sunitha, Nagaraj, Varalaksmi, 2001; Sudhahar *et al.*, 2007b; Sudhahar *et al.*, 2008; Sudhahar, Veena, Varalaksmi, 2008).

The aim of this work was the semi-synthesis of eight lupeol esters, from which five (**5** to **9**) are new ones. The compounds were characterized by Fourier transform infrared (FT-IR), nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectroscopy, CHN analysis and powder X-ray diffractometry (XRD). Moreover, lupeol and the eight lupeol esters were evaluated regarding their antiproliferative *in vitro* potential against a human cell lines panel.

RESULTS AND DISCUSSION

Synthesis and identification of lupeol esters

Lupeol (**1**) was isolated from hexane branch extract of *M. salicifolia* through phytochemical processes as described in the literature (Magalhães *et al.*, 2011). The esters **2** to **9** were obtained reacting **1** with an adequate carboxylic acid and the DIC/DMAP reagents (Figure 1), with yields ranging from 86 to 96%.The ^{1}H and ^{13}C NMR chemical shift assignments of compound **1** (**S3**) were in accordance with the spectral data published by Shahlaei and coworkers (2013).

The structures of lupeol esters (**2** to **9**) were confirmed due to the disappearance of signal at δ_C 71.0, in the ^{13}C NMR spectra (**S4** to **S11**), corresponding to carbon 3 bonds in the hydroxyl group, the presence of signal at ~ δ_C 80.0 associated to C-O-C, together the of the signal at ~ δ_C 171.0 (C=O). The signal associated to C=O group to compound **9** appeared at δ_C 166.10 due to the influence of 3',4'-dimethoxybenzoate group (Mahato, Kundu, 1994). The physical chemical data (IR, ^{1}H and ^{13}C NMR and CHN analysis) of compounds **1** to **9** are described below, as well as the amount obtained (mmol) and percent yield for the esters **2** to **9.**

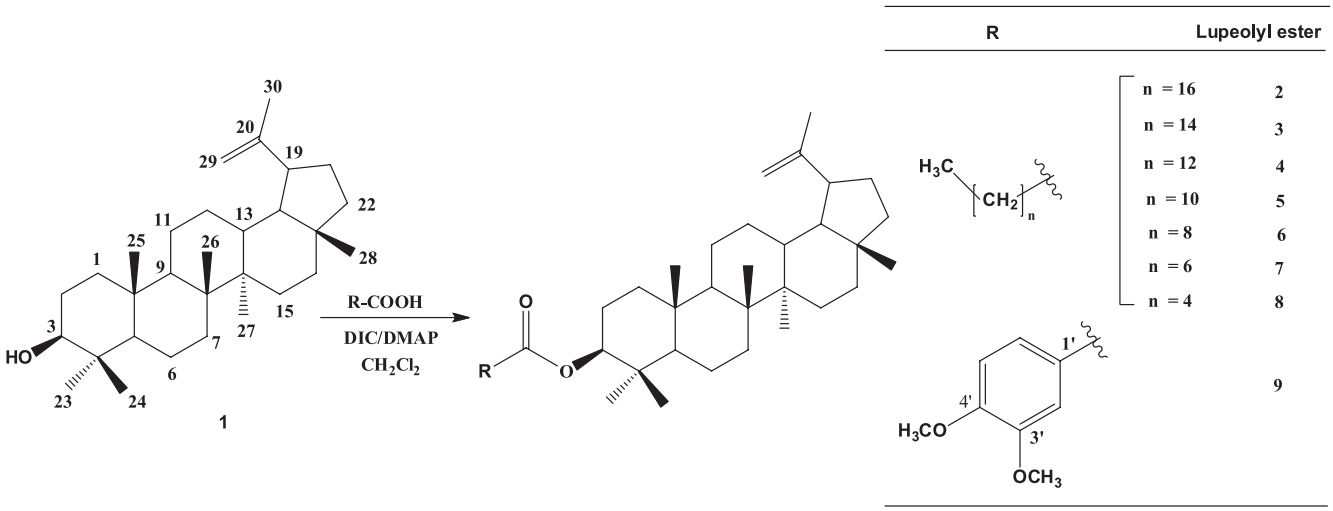

FIGURE 1 - Scheme of lupeol (**1**) esterification using DIC/DMAP reagents, to obtain lupeol esters **2** to **9**.

3β-Lup-20(29)-en-3-ol (lupeol) (**1**): 426 g mol^{-1} [mp 213.8 - 215.2 ºC].
IR (KBr, cm^{-1}): 3550, 3400, 3295, 2920, 2850, 1640 (weak) 1470, 1455, 1440, 1380, 1360, 1140, 1110, 1040, 985, 880.
^{1}H NMR ($CDCl_3$, 200 MHz) δ:4.57 (s, H-29a), 4.68(s, H-29b), 3.21(dd, *J*= 2.0; 6.0 Hz, H-3) ,1.68, 1.00, 0.97, 0.95, 0.83, 0.79, 0.76 (21 H, 7 s, 7 CH_3).
^{13}C NMR ($CDCl_3$, 50 MHz) δ: 38.08 (C-1), 27.43(C-2), 79.05 (C-3), 38.73 (C-4), 55.33 (C-5), 18.34 (C-6), 34.31 (C-7), 40.86 (C-8), 50.47 (C-9), 37.20 (C-10), 20.95 (C-11), 25.17 (C-12), 38.88 (C-13), 42.86 (C-14), 27.47 (C-15), 35.61 (C-16), 43.02 (C-17), 48.34 (C-18), 48.00 (C-19), 150.98 (C-20), 29.87 (C-21), 40.02 (C-22), 28.00 (C-23), 15.37 (C-24), 16.13 (C-25), 16.00 (C-26), 14.57 (C-27), 18.02 (C-28), 109.33 (C-29), 19.32 (C-30).
CHN analysis: Calcd for $C_{30}H_{50}O$: C, 84.44; H, 11.81%. Found: C, 84.49; H, 11.03%.

Lupeol stearate (**2**): 692 g mol^{-1}, [0.85 mmol (86% yield)], (mp 51.7 - 52.7 °C).
IR (KBr, cm^{-1}): 3071, 2915, 2850 (CH), 1727 (C=O), 1640, 1172 (CO-O-C), 882.
^{1}H NMR ($CDCl_3$, 200 MHz) δ:4.68(s, H-29b), 4.58 (s, H-29a), 4.49 (dd, *J*= 4.0; 8.0 Hz, H-3), 1.68, 1.06, 0.94, 0.90, 0.88, 0.83, 0.79 (21H, 7 s, 7 CH_3).
^{13}C NMR ($CDCl_3$, 50 MHz) δ: 38.40 (CH_2, C-1), 23.74 (CH_2, C-2), 80.61 (C,C-3), 37.83 (C,C-4), 55.37 (CH,C-5), 18.20 (CH_2, C-6), 34.20 (CH_2, C-7), 40.84 (C, C-8), 50.33 (CH, C-9), 37.08 (C, C-10), 20.94 (CH_2, C-11), 25.10 (CH_2, C-12), 38.04 (CH, C-13), 42.9 (C, C-14), 27.43 (CH_2, C-15), 35.57 (CH_2, C-16), 42.82 (C, C-17), 48.28 (C, C-18), 48.00 (C,C-19), 150.94 (C, C-20), 29.70 (CH_2, C-21), 40.00 (CH_2, C-22), 27.97 (CH_3, C-23), 16.57 (CH_3, C-24), 16.17 (CH_3, C-25), 15.97 (CH_3, C-26), 14.51 (CH_3, C-27), 18.00 (CH_3, C-28), 109.36 (CH_2, C-29), 19.28 (CH_3, C-30), 173.72 (C, C-1'), 34.86 (CH_2, C-2'), 25.17 (CH_2, C-3'), 29.26 (CH_2, C-4'), 29.27 (CH_2, C-5'), 29.38 (CH_2, C-6'), 29.59 (CH_2, C-7'), 14.13 (CH_2, C-8'), 29.82 (CH_2, C-9'), 29.70 (CH_2, C-10'), 29.70 (CH_2, C-11'), 29.70 (CH_2, C-12'), 29.70 (CH_2, C-13'), 29.59 (CH_2, C-14'), 29.47 (CH_2, C-15'), 31.94 (CH_2, C-16'), 22.70 (CH_2, C-17'), 14.13 (CH_3, C-18') .
CHN analysis: Calcd for $C_{48}H_{84}O_2$: C, 83.17; H, 12.21%. Found: C, 82.97; H, 13.41%.

Lupeol palmitate (**3**): 664 g.mol^{-1}, [0.90 mmol (90% yield)], (mp 52.0 - 56.0 ºC).
IR (KBr, cm^{-1}): 3071, 2915, 2850 (CH), 1726 (C=O), 1641, 1171 (CO-O-C), 881.
^{1}H NMR ($CDCl_3$, 200 MHz) δ:4.57 (s, H-29a), 4.68(s, H-29b), 4.48 (dd, *J*= 6.0; 12.0 Hz, H-3), 1.68, 1.03, 0.94, 0.88, 0.85, 0.84, 0.79 (21H, 7 s, 7 CH_3).
^{13}C NMR ($CDCl_3$, 50 MHz) δ: 38.39 (CH_2, C-1), 23.76 (CH_2, C-2), 80.63 (C, C-3), 37.85 (C, C-4), 55.39 (CH, C-5), 18.22 (CH_2, C-6), 34.22 (CH_2, C-7), 40.87 (C, C-8), 50.35 (CH, C-9), 37.10 (C, C-10), 20.96 (CH_2, C-11), 25.11 (CH_2, C-12), 38.01 (CH, C-13), 42.84 (C, C-14), 27.45 (CH_2, C-15), 35.59 (CH_2, C-16), 43.01 (C ,C-17) , 48.30 (C, CH-18), 48.32 (C, C-19) , 150.98 (C, C-20), 29.84 (CH_2, C-21), 40.02 (CH_2, C-22), 27.99 (CH_3, C-23), 16.59 (CH_3, C-24), 16.19 (CH_3, C-25), 15.99 (CH_3, C-26), 14.54 (CH_3, C-27), 18.02 (CH_3, C-28), 109.38 (CH_2, C-29), 19.30 (CH_3, C-30), 173.74 (C, C-1'), 34.88 (CH_2, C-2'), 25.01 (CH_2, C-3'), 29.39 (CH_2, C-4'), 29.49 (CH_2, C-5'), 29.70 (CH_2, C-6'), 29.61 (CH_2, C-7'), 29.61 (CH_2, C-8'), 29.61 (CH_2, C-9'), 29.61 (CH_2, C-10'), 29.61 (CH_2, C-11'), 29.61 (CH_2, C-12'), 29.29 (CH_2, C-13'), 31.95 (CH_2, C-14'), 22.72 (CH_2, C-15'), 14.15 (CH_3, C-16').
CHN analysis: Calcd for $C_{46}H_{80}O_2$: C, 83.07; H, 12.12%. Found: C, 83.25; H, 12.64%.

Lupeol miristate (**4**): 636 g mol^{-1}, [0.95 mmol (95% yield)], (mp 84.5 - 86.8 °C).
IR (KBr, cm^{-1}): 2953, 2850(CH), 1728 (C=O), 1171(CO-O-C), 881.
^{1}H NMR ($CDCl_3$, 200 MHz) δ:4.57 (s, H-29a), 4.68 (s, H-29b), 4.48 (dd, *J*= 6.0; 12.0 Hz, H-3), 1.68, 1.03, 0.94, 0.91, 0.88, 0.84, 0.79 (21H, 7 s, 7 CH_3).
^{13}C NMR ($CDCl_3$, 50 MHz) δ: 38.36 (CH_2, C-1), 23.74 (CH_2, C-2), 80.61 (C, C-3), 37.83 (C, C-4), 55.37(CH, C-5), 18.20 (CH_2, C-6), 34.20 (CH_2, C-7), 40.84 (C, C-8), 50.32 (CH, C-9), 37.08 (C, C-10), 20.93 (CH_2, C-11), 25.08 (CH_2, C-12), 38.03 (CH, C-13), 42.82 (C, C-14), 27.43 (CH_2, C-15), 35.56 (CH_2, C-16), 43.00 (C, C-17), 48.28 (C, C-18), 48.01 (C, C-19), 150.98 (C, C-20), 29.83 (CH_2, C-21), 40.00 (CH_2, C-22), 27.97 (CH_3, C-23), 16.58 (CH_3, C-24), 16.17 (CH_3, C-25), 15.97 (CH_3, C-26), 14.52 (CH_3, C-27), 18.00 (CH_3, C-28), 109.36 (CH_2, C-29), 19.28 (CH_3, C-30), 173.74 (C, C-1'), 34.87 (CH_2, C-2'), 25.18 (CH_2, C-3'), 29.48 (CH_2, C-4'), 29.37 (CH_2, C-5'), 29.65 (CH_2, C-6'), 29.60 (CH_2, C-7'), 29.60 (CH_2, C-8'), 29.60 (CH_2, C-9'), 29.60 (CH_2, C-10'), 29.27 (CH_2, C-11'), 31.93 (CH_2, C-12'), 22.70 (CH_2, C-13'), 14.14 (CH_2, C-14').
CHN analysis: Calcd for $C_{44}H_{76}O_2$: C, 82.95; H, 12.02%. Found: C, 83.13; H, 12.49%.

Lupeol laurate **(5)**: 608 g mol^{-1}, [0.89 mmol (89% yield)], (mp 93.4 - 95.1 °C).
IR (ATR, cm^{-1}): 2923, 2853, 1727(C=O), 1176(CO-O-C), 877.

^{1}H NMR ($CDCl_3$, 200 MHz) δ:4.57 (s, H-29a), 4.68 (s, H-29b), 4.47 (m, H-3), 1.68, 1.02, 0.94, 0.88, 0.85,b0.84, 0.79 (21H, 7 s, 7 CH_3).
^{13}C NMR ($CDCl_3$, 50 MHz) δ: 38.37 (CH_2, C-1), 23.75 (CH_2, C-2), 80.60 (C, C-3), 37.83 (C, C-4), 55.37 (CH, C-5), 18.20 (CH_2, C-6), 34.21 (CH_2, C-7), 40.84 (C, C-8), 50.33 (CH, C-9), 37.08 (C, C-10), 20.94 (CH_2, C-11), 25.17 (CH_2, C-12), 38.04 (CH, C-13), 42.82 (C, C-14), 27.43 (CH_2, C-15), 35.57 (CH_2, C-16), 43.00 (C, C-17), 48.28 (CH, C-18), 48.01 (C, C-19), 150.94 (C, C-20), 29.82 (CH_2, C-21), 40.00 (CH_2, C-22), 27.97 (CH_3, C-23), 16.57 (CH_3, C-24), 16.17 (CH_3, C-25), 15.97 (CH_3, C-26), 14.52 (CH_3, C-27), 18.00 (CH_3, C-28), 109.37 (CH_2, C-29), 19.28 (CH_3, C-30), 173.71 (C, C-1'), 34.86 (CH_2, C-2'), 25.17 (CH_2, C-3'), 31.91 (CH_2, C-4'), 22.69 (CH_2, C-5'), 29.44 (CH_2, C-6'), 29.59 (CH_2, C-7'), 27.42 (CH_2, C-8'), 29.26 (CH_2, C-9'), 31.90 (CH_2, C-10'), 29.81 (CH_2, C-11'), 14.12 (CH_2, C-12').
CHN analysis: Calcd for $C_{42}H_{72}O_2$: C, 82.83; H, 11.92%. Found: C, 83.07; H, 13.09%.

Lupeol caprate (**6**): 580 g mol^{-1}, [0.92 mmol (92% yield)], (mp 92.5 - 93.8 °C).
IR (ATR, cm^{-1}): 2928, 2851, 1728(C=O), 1175(CO-O-C), 881.
^{1}H NMR ($CDCl_3$, 200 MHz) δ:4.57 (s, H-29a), 4.68(s, H-29b), 4.47 (m, H-3), 1.68, 1.02, 0.94, 0.88, 0.85, 0.84, 0.79 (21H, 7 s, 7 CH_3).
^{13}C NMR ($CDCl_3$, 50 MHz) δ: 38.36 (CH_2, C-1), 23.75 (CH_2, C-2), 80.60 (C, C-3), 37.83 (C, C-4), 55.37 (CH, C-5), 18.20 (CH_2, C-6), 34.20 (CH_2, C-7), 40.84 (C, C-8), 50.33 (CH, C-9), 37.08 (C, C-10), 20.94 (CH_2, C-11), 25.17 (CH_2, C-12), 38.04 (CH, C-13), 42.82 (C, C-14), 27.43 (CH_2, C-15), 35.57 (CH_2, C-16), 43.00 (C, C-17), 48.28 (CH, C-18), 48.00 (C, C-19), 150.94 (C, C-20), 29.82 (CH_2, C-21), 40.00 (CH_2, C-22), 27.97 (CH_3, C-23), 16.57 (CH_3, C-24), 16.17 (CH_3, C-25), 15.97 (CH_3, C-26), 14.52 (CH_3, C-27), 18.00 (CH_3, C-28), 109.36 (CH_2, C-29), 19.28 (CH_3, C-30), 173.70 (C, C-1'), 34.87(CH_2, C-2'), 25.17 (CH_2, C-3'), 29.26 (CH_2, C-4'), 29.19 (CH_2, C-5'), 29.43 (CH_2, C-6'), 29.43 (CH_2, C-7'), 31.86 (CH_2, C-8'), 29.82 (CH_2, C-9'), 14.11 (CH_2, C-10').
CHN analysis: Calcd for $C_{40}H_{68}O_2$: C, 82.69; H, 11.80%. Found: C, 82.77; H, 13.03%.

Lupeol caprilate (**7**): 552 g mol^{-1}, [0.96 mmol (96% yield)], (mp 145.0-145.7 °C).
IR (KBr, cm^{-1}): 1727 (C=O); 1179 (CO-O-C), 2927, 2852 (CH).
^{1}H NMR ($CDCl_3$, 200 MHz) δ:4.57 (s, H-29a), 4.68(s, H-29b), 4.48 (dd, *J*= 6.0; 8.0 Hz, H-3), 1.68, 1.03, 0.94, 0.87, 0.85, 0.84, 0.79 (s, 21H, 7 s, 7 CH_3).
^{13}C NMR ($CDCl_3$, 50 MHz) δ: 38.36 (CH_2, C-1), 23.73 (CH_2, C-2), 80.60 (C, C-3), 38.02 (C, C-4), 55.36 (CH, C-5), 18.19 (CH_2, C-6), 34.20 (CH_2, C-7), 40.83 (C, C-8), 50.32 (CH, C-9), 37.07 (C, C-10), 20.93 (CH_2, C-11), 25.16 (CH_2, C-12), 38.02 (CH, C-13), 42.82 (C, C-14), 27.42 (CH_2, C-15), 35.56 (CH_2, C-16), 42.98 (C, C-17), 48.27 (CH, C-18), 48.00 (C, C-19), 150.95 (C, C-20), 29.81 (CH_2, C-21), 40.83 (CH_2, C-22), 27.95 (CH_3, C-23), 16.56 (CH_3, C-24), 16.16 (CH_3, C-25), 15.96 (CH_3, C-26), 14.51 (CH_3, C-27), 18.19 (CH_3, C-28), 109.35 (CH_2, C-29), 19.27 (CH_3, C-30), 173.70 (C, C-1'), 34.85 (CH_2, C-2'), 25.16 (CH_2, C-3'), 29.13 (CH_2, C-4'), 29.13 (CH_2, C-5'), 31.67 (CH_2, C-6'), 22.59 (CH_2, C-7'), 14.06 (CH_2, C-8').
CHN analysis: Calcd for $C_{38}H_{64}O_2$: C, 82.55; H, 11.67%. Found: C, 82.68; H, 12.96%.

Lupeol caproate (**8**): 524 g mol^{-1}, [0.87 mmol (87% yield)], (mp 156.4 - 159.7 °C).
IR (KBr, cm^{-1}): 2936, 2858, 1727(C=O), 1180(CO-O-C), 877.
^{1}H NMR ($CDCl_3$, 200 MHz) δ:4.58 (d, J=2.0Hz, H-29a), 4.68 (d, J=2.0Hz, H-29b), 4.47 (dd, *J*= 4.0; 6.0 Hz, H-3), 1.68, 1.03, 0.94, 0.85, 0.84, 0.83, 0.79 (21H, 7 s, 7 CH_3)
^{13}C NMR ($CDCl_3$, 50 MHz) δ: 38.37 (CH_2, C-1), 23.74 (CH_2, C-2), 80.62 (C, C-3), 37.83 (C, C-4), 55.37 (CH, C-5), 18.20 (CH_2, C-6), 34.21 (CH_2, C-7), 40.85 (C, C-8), 50.33 (CH, C-9), 37.08 (C, C-10), 20.94 (CH_2, C-11), 25.09 (CH_2, C-12), 38.04 (CH, C-13), 42.83 (C, C-14), 27.43 (CH_2, C-15), 35.57 (CH_2, C-16), 42.99 (C, C-17), 48.28 (CH, C-18), 48.00 (C, C-19), 150.95 (C, C-20), 29.83 (CH_2, C-21), 39.99 (CH_2, C-22), 27.96 (CH_3, C-23), 16.56 (CH_3, C-24), 16.17 (CH_3, C-25), 15.97 (CH_3, C-26), 14.52 (CH_3, C-27), 18.00 (CH_3, C-28), 109.36 (CH_2, C-29), 19.28 (CH_3, C-30), 173.71 (C, C-1'), 34.81 (CH_2, C-2'), 24.83 (CH_2, C-3'), 31.33 (CH_2, C-4'), 22.32 (CH_2, C-5'), 13.92 (CH_3, C-6').
CHN analysis: Calcd for $C_{36}H_{60}O_2$: C, 82.38; H, 11.52%. Found: C, 82.66; H, 12.72%.

Lupeol 3',4'-dimethoxybenzoate (**9**): 590 g.mol^{-1}, [0.90 mmol (90% yield)], (mp 243.6 - 245.3 °C).
IR (KBr, cm^{-1}): 2944, 2857, 1704, 1267, 1247, 968, 761.
^{1}H NMR ($CDCl_3$, 200 MHz) δ: 7.70 (d, J=10.0 Hz, H-6´), 7.56 (s, H-2´), 6.90 (d, J=8.0Hz, H-5´) 4.69 (t, H-29a,b), 4.58 (s, H-3), 3.93 (s, H-9´, H-10´), 1.68, 1.05, 0.99, 0.97, 0.92, 0.90, 0.80 (21H, 7 s, 7 CH_3).
^{13}C NMR ($CDCl_3$, 50 MHz) δ: 38.40 (CH_2,C-1), 25.11(CH_2, C-2), 81.38 (C, C-3), 38.22 (C, C-4), 55.44 (CH, C-5), 18.24 (CH_2, C-6), 34.22 (CH_2, C-7), 40.88

(C, C-8), 50.36 (CH, C-9), 37.14 (C, C-10), 20.98 (CH_2, C-11), 23.83 (CH_2, C-12), 38.06 (CH, C-13), 42.86 (C, C-14), 27.45 (CH_2, C-15), 35.58 (CH_2, C-16), 43.00 (C, C-17), 48.29 (CH, C-18), 48.02 (C, C-19), 150.95 (C, C-20), 29.84 (CH_2, C-21), 40.01 (CH_2, C-22), 28.14 (CH_3, C-23), 16.01 (CH_3, C-24), 16.81 (CH_3, C-25), 16.20 (CH_3, C-26), 14.56 (CH_3, C-27), 18.02 (CH_3, C-28), 109.38 (CH_2, C-29), 19.30 (CH_3, C-30), 166.10 (C, C-1'), 152.79 (C, C-2'), 112.03 (CH, C-3'), 148.59 (C, C-4'), 150.95 (C, C-5'), 110.21 (CH, C-6'), 123.35 (CH, C-7'), 55.94 (CH_3, C-8'), 55.94 (CH_3, C-9').
CHN analysis: Calcd for $C_{39}H_{58}O_4$: C, 79.28; H, 9.89%. Found: C, 79.33; H, 10.56%.

XRD analysis of compound **1** revealed its needle shape and established a structure in which the carbon atoms distribution (Figure 2) is in accordance with the ^{13}C NMR data.

For the XRD experiments, the material was homogenously spread over the sample holder under spinning to prevent preferred orientation and minimize rugosity effects over the exposed surface. The small amount submitted to the XRD, few milligrams, was composed essentially of polycrystalline material. Single crystals were not identified or isolated from the synthetic material. So, detailed crystallographic data were provided only for the isolated lupeol. For lupeol (**1**), the angles are 90.0 due to the special positions on tetragonal P43 space group symmetric restrictions. Other details of refinements and X-ray diffraction experimental data are summarized in Table I. Due to the small amount of esters (**2** to **9**), all fittings were obtained at P-1 space group that safely allowed us to index all peaks. After extracting and fitting, all peaks in space group P-1 were searched for more symmetric space group based on the Bragg systematic absences. More symmetric space groups were achieved for compounds **1**, **7** and **8**. The remaining ones have not shown any symmetric description based on the systematic Bragg absences. The powder XRD data of lupeol esters **2** to **9** (Table I) were consistent with the ^{13}C NMR data of each one indicating the tendency of the compounds to be in the crystalline state. The XRD experiment was considered as an excellent tool to determine the structure of lupeol and its esters in solid state.

Cell proliferation assays

All the compounds were tested for proliferation of human cancer cells. Doxorubicin (anthracycline) used as positive control is a chemotherapy drug that decreases or stops the growth of cancer cells. The activity of doxorubicin involves blocking the enzyme called topo isomerase 2 that cancer cells need to replicate and grow. Lupeol was inactive (GI_{50}>250 µg/mL) in the experimental condition while lupeol esters **2**-**4** and **7**-**9** showed a cytostatic effect on colorectal adenocarcinoma (HT-29) and chronic myelogenous leukemia (K-562) cell lines (Table II).

The introduction of a long alkyl side chain (**2**) in lupeol resulted in a cytostatic effect on the colorectal adenocarcinoma (HT29) cell line (GI_{50} = 97.81 µg/mL). This effect increased by reducing the length of the alkyl chain, from C16 (**2**) to C12 (**4**), resulting in the best effect (GI_{50} = 1.74 µg/mL). However, the continuous

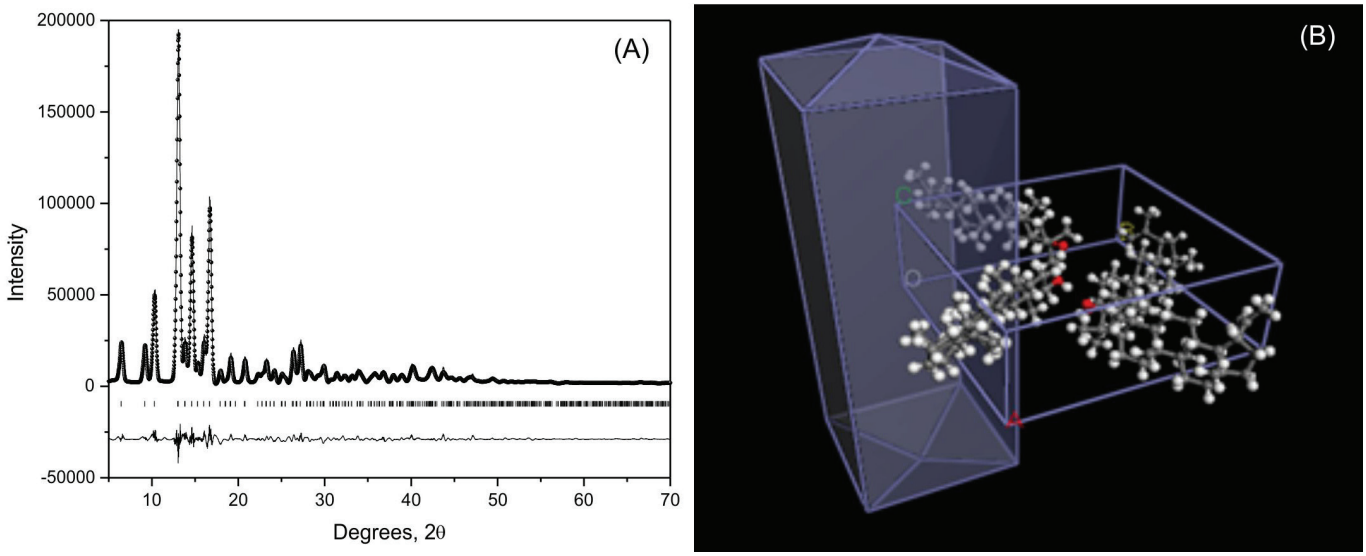

FIGURE 2 - Powder X-ray diffraction (XRD) data: (A) lupeol difratogram; (B) lupeol chemical structure, found in solid with a needle shape.

TABLE I - Lupeol (**1**) and its esters (**2** to **9)** lattice parameters obtained by Rietveld fitting of the powder X-ray diffraction

Compounds	*a* (nm)	*b* (nm)	*c* (nm)	α (⁰)	β (⁰)	γ (⁰)	Space Group
1	19.513±0.005	19.513±0.005	7.383±0.002	90.0	90.0	90.0	P43
2	*	*	*	*	*	*	*
3	7.37±0.04	16.8±0.1	17.1±0.1	98.64±0.01	106.41±0.01	90.35±0.01	P-1
4	7.68±0.01	11.08±0.01	14.55±0.01	89.30±0.01	83.82±0.01	77.33±0.01	P-1
5	6.98±0.07	8.73±0.09	9.8±0.1	87.52±0.03	88.33±0.02	89.12±0.03	P-1
6	9.56±0.01	11.32±0.01	21.22±0.01	102.63±0.01	95.65±0.01	92.66±0.01	P-1
7	8.03±0.02	18.55±0.06	12.74±0.04	90.0	96.68±0.01	90.0	P 121
8	25.3±0.2	8.15±0.06	21.6±0.2	90.0	119.63±0.02	90.0	C121
9	10.39±0.06	11.60±0.06	13.92±0.07	98.50±0.01	91.80±0.01	105.87±0.01	P-1

TABLE II - Concentration (μg/mL) of lupeol (**1**) and its esters (**2 - 9**) necessary to inhibit 50% cell growth (GI_{50})

Compound tested	Cell lines							
	U251	NCI-ADR/ RES	786-0	NCI-H460	PC-3	OVCAR-03	HT29	K562
Doxo	<0.025	25	0.038	<0.025	0.025	0.23	0.026	>25
1	*	*	*	*	*	*	*	*
2	*	*	*	*	*	*	97.81	0.35
3	*	*	*	250	*	*	35.94	<0.25
4	*	*	*	*	*	*	1.74	0.41
5	*	*	*	*	*	*	*	*
6	*	*	*	*	*	*	*	*
7	*	*	*	*	*	*	*	<0.25
8	*	*	*	*	*	*	250	<0.25
9	*	*	*	*	*	*	0.95	<0.25

Key: * = GI_{50}>250 μg/mL; Doxo, doxorubicine (positive control); 3β-Lup-20(29)-en-3-ol (lupeol) (**1**); lupeol stearate (**2**); lupeol palmitate (**3**); lupeol miristate (**4**); lupeol laurate (**5**); lupeol caprate (**6**); lupeol caprilate (**7**); lupeol caproate (**8**); lupeol 3',4'-dimethoxybenzoate (**9**). Human tumor cell lines: U251 (glioma), NCI-ADR/RES (ovarian expressing the resistance phenotype for adryamycin), 786-0 (kidney), NCI-H460 (lung, non-small cells), PC-3 (prostate), OVCAR-03 (ovarian), HT-29 (colon), and K-562 (chronic myelogenous leukemia).

reduction in the chain length (from C10 to C4, compounds **5**-**8**) afforded inactive compounds (GI_{50}>250 μg/mL). Moreover, lupeol palmitate (**3**) showed a selective growth inhibition effect on erythromyeloblastoid leukemia (K-562) cells in a concentration-dependent way (Figure S2). Previous studies have shown that the palmitic acid is active against leukemic cells (Harada *et al.*, 2002). Probably, the activity observed for lupeol palmitate is due to the fatty moiety. Thus, compound **3** could be considered as a prototype for the development of new anticancer drugs to be used in leukemia treatment. For the aryl lupeol ester (**9**) it was seen a quite similar cytostatic effect (GI_{50}=0.95 μg/mL) to that observed for ester **4**. On the other hand, the side chain length seemed not to be as influent against chronic myelogenous leukemia (K-562) cells as for antiproliferative activity against HT-29. In this regard, only compounds **5** (with ten carbon in side chain) and **6** (with eight carbons in side chain) were not able to inhibit K-562 cell proliferation up to 250 μg/mL (Figure S2, Table II). The esters **4** and **9** showed a selective cytostatic effect with low GI_{50} values (Figure S2), therefore, these compounds represent a promising prototype for the development of new anticancer drugs.

For the aryl lupeol ester (**9**) it was seen a quite similar cytostatic effect (GI_{50} = 0.95 μg/mL) than observed for ester **4**.

CONCLUSION

The esters **2** to **9** were obtained using lupeol, an adequate carboxylic acid and DIC/DMAP reagents, with yields ranging from 86 to 96%. The esters **5** to **9** were new compounds. The XDR method was an excellent tool to determine the structure of lupeol and its esters in solid state. Lupeol esters **3**, **4** and **9** showed a selective cytostatic effect with low GI_{50} values, representing a promising prototype for the development of new anticancer drugs.

EXPERIMENTAL SECTION

General experimental procedures

Melting points (mp) (uncorrected) were determined using a Mettler FP 80 HT apparatus. ^{13}C NMR spectra were obtained on a Bruker *Avance* DRX 400 or on Bruker DPX 200 spectrometers. The sample was dissolved in $CDCl_3$ and TMS was used as internal standard ($\delta_C = 0$). IR spectra were recorded on a FITR–Perkin-Elmer, Spectrum One SN 74759 spectrophotometer. Powder X-ray diffraction (XRD) data were collected in an XRD-7000 diffractometer (Shimadzu, Japan) under 40 kV, 30 mA, using Cu Kα (λ = 1.54056 Å) equipped with a polycapillary focusing optics under parallel geometry coupled with a graphite monochromator, scanned over an angular range of 4−70° (2θ) with a step size of 0.01° (2θ) and a time constant of 5 $s.step^{-1}$. The sample holder was submitted to a spinning of 30 cycles per minute to minimize rugosity effects and to reduce any eventual preferred orientation. The lattice parameters were extracted and fitted by Rietveld fitting analysis. CHN analyses were performed in a Perkin Elmer, Series II, CHNS/O Analyzer. Classical chromatographic column (CC) was carried out using silica gel 60 (Merck, 70-230 Mesh). TLC was obtained using pre-coated silica gel plates, and the detection was visualized by spraying the plates with solution (1:1) of vanillin (ethanol 1 % solution w/v) in perchloric acid (3% aqueous solution v/v), in accordance with Wagner and Bladt (1996).

Plant material

Maytenus salicifolia Reissek (Celastraceae) was collected at 'Serra de Ouro Branco', a mountain located in the Ouro Branco City region, Minas Gerais (MG) state, Brazil. The plant was identified by Dr. Rita Maria Carvalho-Okano, Botanist of the Universidade Federal de Viçosa, MG, Brazil. A voucher specimen of *M. salicifolia* was deposited (Nº. OUPR-18094) at the *Herbarium* José Badini of the Universidade Federal de Ouro Preto, MG, Brazil.

Isolation of lupeol and synthesis of esters

The isolation of lupeol was reported by *Magalhães* and coworkers (2011). For the esters synthesis, the following sequence was carried out for the reactions: to 1.0 mmol of lupeol (**1**), **x** mmol of carboxylic acid and **y** mmol of 4-(dimethylamino)pyridine (DMAP) in 7.0 mL of dry dichloromethane were added (Table I). After cooling down to 0 °C and under constant magnetic stirring, **z** mmol of *N,N'*-diisopropylcarbodiimide (DIC) was carefully added. Then, the reaction mixture was maintained under magnetic stirring, at room temperature, for 2 to 48 hours depending on the carboxylic acid used as reagent. The reaction time was monitored by TLC using $CHCl_3$-MeOH (9.5:0.5) as mobile phase. The reaction conditions of carboxylic acid with lupeol [**1**, (1.0 mmol)] and DIC/DMAP to obtain the lupeol esters **2** to **9** (Figure 1) are presented in Table SI.

At the end of the reaction, the dichloromethane was recovered in a rotator evaporator and the residual material obtained from each esterification reaction was purified by chromatographic column eluted with $CHCl_3$. The lupeol esters **2** to **4** (Figure 1) were obtained as a white waxy material while lupeol esters **5** to **9** were obtained as a white amorphous solid.

Characterization of compounds

The structure of lupeol and its synthesized esters were initially characterized by IR, NMR (^1H, ^{13}C) and CHN data. The spectral results were carefully compared with data available in the literature (Mahato, Kundu, 1994). Then, the structure of each compound was fitted through powder XRD. Thus, compound **1** (or ester **2** to **9**) was reduced to a very fine powder and deposited as a film suspension in a Zero Field Sample Holder (ZFSH) composed by polished SiC in a 3° angle mount to reduce background contributions for the X-ray diffraction experiment. The powder indexing tool used was Conograph (Oishi-Tomiyasu, 2012) for cell and space group determination, followed by Pareto optimization and Rietveld with energies fitting of the structure. The peaks were searched and fitted with a David-Voight approximation peak profile, performing both modified Pawley and Rietveld with energy refinements to optimize powder diffraction parameters and crystal structure, so that the best possible agreement between simulated and experimental powder pattern was achieved. Lattice parameters were expressed in nanometers (nm) and the angles in degrees (0). The fitted uncertainties were listed with the significant figures obtained. Due to the

small amount of material (lupeol esters), all fittings were obtained at P-1 space group, which allowed us to index all peaks safely. To search for more symmetric Space Group occurrences more natural extracted material would be necessary to increase low intensity peaks that may help search for more symmetry in all diffractograms. After extracting and fitting all peaks in space group P-1, a search was performed for more symmetric space groups based on the Bragg systematic absences. Details of refinements and experimental data of X-ray diffraction are summarized in Table I.

Antiproliferative activity

Human cell lines

Eight human tumor cell lines were used: U-251 (glioma), NCI-ADR/RES (ovarian expressing the resistance phenotype for adryamycin), 786-0 (kidney), NCI-H460 (lung, non-small cells), PC-3 (prostate), OVCAR-03 (ovarian), HT-29 (colon), and K-562 (erythromyeloblastoid leukemia). The eight human tumor cell lines were provided by the Frederick Cancer Research & Development Center, National Cancer Institute, Frederick, MA, USA. The cells were grown in RPMI 1640 Medium (GIBCO BRL) supplemented with 5% fetal bovine serum (FBS) (GIBCO BRL) and penicillin/streptomycin mixture (1000 U/mL: 1000 µg/mL, 1.0 mL/L RPMI) at 37 ^{o}C in a 5% CO_2 atmosphere.

Sample preparation

Aliquots (5.0 mg) of lupeol and its esters **2** to **9** were initially diluted in DMSO (50 µL) followed by the addition of 950 µL of RPMI 1640/FBS 5% (working solution). The solutions were then diluted in RPMI 1640/FBS 5% in order to obtain the final concentrations. DMSO final concentrations (≤ 0.25%) in culture medium did not affect the cell viability.

Antiproliferative assay

Cells in 96-well microplates (100 µL cells/well, inoculation density from 3 to 7 x 10^4 cell/mL) were exposed for 48 h to crescent concentrations (0.25, 2.5, 25.0, and 250.0 µg/mL, in triplicate) of **1** and its esters **2** to **9** at 37 ^{0}C in a 5% CO_2 atmosphere. Doxorubicin chloridrate (0.1mg/mg; Europharma) was used as positive control (0.025, 0.25, 2.5 and 25 µg/ml). Before (T0 plate) and after sample addition (T1 plates), cells were fixed with 50% trichloroacetic acid (50 µL/well). Cellular proliferation was determined by the spectrophotometric quantification (540 nm) of cellular protein content using sulforhodamine B assay (Monks *et al*., 1991). Using the concentration-response curve for each cell line, GI_{50} (concentration that inhibits cell growth by 50%) was determined through non-linear regression analysis using the software ORIGIN 8.0 (Origin Lab Corporation) (Dos Santos *et al.*, 2015; Da Silva *et al.*, 2015).

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