1 **Isololiolide, a carotenoid metabolite isolated from the brown alga *Cystoseira***

2 ***tamariscifolia*, is cytotoxic and able to induce apoptosis in hepatocarcinoma cells**

3 **through caspase-3 activation, decreased Bcl-2 levels, increased p53 expression and**

4 **PARP cleavage**

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ABSTRACT

Background: Brown macroalgae have attracted attention because they display a wide range of biological activities, including antitumoral properties. In a previous screen we isolated isololiolide from *Cystoseira tamariscifolia* for the first time.

Purpose: To examine the therapeutical potential of isololiolide against tumor cell lines.

Methods/Study design: The structure of the compound was established and confirmed by 1D and 2D NMR as well as HRMS spectral analysis. The *in vitro* cytotoxicity was analyzed by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in tumoral as well as in non-tumoral cell lines. Cell cycle arrest and induction of apoptosis were assessed by flow cytometry. Alteration of expression levels in proteins important in the apoptotic cascade was analyzed by western blotting.

Results: Isololiode was isolated for the first time from the brown macroalga *Cystoseira tamariscifolia*. Isololiolide exhibited significant cytotoxic activity against three human tumoral cell lines, namely hepatocarcinoma HepG2 cells, whereas no cytotoxicity was found in non-malignant MRC-5 and HFF-1 human fibroblasts. Isololiode completely disrupted the HepG2 normal cell cycle and induced significant apoptosis. Moreover, Western blot analysis showed that isololiode altered the expression of proteins that are important in the apoptotic cascade, namely increasing PARP cleavage and p53 expression while decreasing procaspase-3 and Bcl-2 levels.

Conclusion: Isololiolide isolated from *C. tamariscifolia* is able to exert a selective cytotoxic activity on hepatocarcinoma HepG2 cells as well as induce apoptosis through the modulation of apoptosis-related proteins.

Keywords: Marine natural product; *Cystoseira*; Isololiolide; Carotenoid metabolite; Cell cycle; Apoptosis

57 *Abbreviations:* 1D and 2D NMR, one-dimensional and two-dimensional nuclear

58 magnetic resonance; ^{1}H and ^{13}C NMR, proton and carbon-13 nuclear magnetic resonance;

59 ANOVA, analysis of variance; Bak, Bcl-2 homologous antagonist killer; Bax, Bcl-2-

60 associated X protein; Bcl-2, B-cell lymphoma 2; CDCl$_3$, deuterated chloroform; CNT,

61 compound-induced cytotoxicity on non-tumoral cells; CT, compound-induced

62 cytotoxicity on tumoral cells; DEPT, distortionless enhancement by polarization transfer;

63 DMEM, Dulbecco's Modified Eagle's medium; DMSO, dimethyl sulfoxide; ECL,

64 enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; EtOAc, ethyl

65 acetate; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HCC, hepatocellular

66 carcinoma; HPLC, high performance liquid chromatography; HRESIMS, high-resolution

67 electrospray ionisation mass spectrometry; HRMS, high resolution mass spectrometry;

68 HRP, horseradish peroxidase; HSD, honest significant difference; IC$_{50}$, half maximal

69 inhibitory concentration; *m/z*, mass-to-charge ratio; MeOH, methanol; MTT, 3-(4,5-

70 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NP-40, nonidet P-40; p53,

71 protein 53; PARP, poly (ADP-ribose) polymerase; PI, propidium iodide; RPMI, Roswell

72 Park Memorial Institute medium; SARs, structure-activity relationships; SDS, sodium

73 dodecyl sulphate; SEM, standard error of mean; SiO$_2$, silica; TLC, thin layer

74 chromatography; TMS, tetramethylsilane; WHO, World Health Organization.

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Introduction

Cancer is a major public health problem with an estimated prevalence of about 3% in Europe, increasing to 15% at old age. Moreover, cancer related deaths are estimated to increase to over 11 million in 2030 (WHO, 2010). Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide, after lung and stomach cancer (Ferenci et al. 2010). The current therapeutics used for HCC treatment involves surgical resection, transplantation and/or systemic chemotherapy; however, surgery and transplantation may not be appropriate for many patients and chemotherapy often fails (Liu et al. 2014). Chemotherapy is also constrained by its toxicity, significant resistance to available chemotherapeutic agents and side effects, including neutropenia and myelosuppression (Chau et al. 2006). Current studies involved in developing effective cancer prevention approaches have focused on the use of bioactive natural agents that may have less adverse effects and can exert selective cytoxicity against cancer cells (Ghate et al. 2014).

The chemical and biological diversity of the marine environment is immeasurable and therefore is an extraordinary resource for the discovery of novel anticancer drugs. Brown algae are a rich source of secondary metabolites displaying a wide variety of bioactivities with important features for pharmaceutical purposes. *Cystoseira tamariscifolia* has demonstrated interesting biological activities such as antibacterial, antifungal, antiprotozoal, cell division inhibition, anti-inflammatory, antioxidant and cytotoxic properties (Bennamara et al. 1999, Spavieri et al. 2010, Lopes et al. 2012, Andrade et al. 2013). These properties have been ascribed to the presence of different classes of molecules that were identified in *C. tamariscifolia*, such as phlorotannins (fucophloroethol, fucodiphloroethol, fucotriphloroethol, 7-phloroeckol, phlorofucofuroeckol and bieckol/dieckol), phloroglucinol, proline, β-sitosterol,

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106 fucosterol, and diverse fatty acids (Ferreres et al. 2012, Andrade et al. 2013, Vizetto-

107 Duarte et al. 2015). As *C. tamariscifolia* extracts have previously demonstrated cytotoxic

108 potential, in this study we describe the identification of isololiolide, a known carotenoid

109 metabolite, as a selective cytotoxic compound that was isolated from the brown

110 macroalga *Cystoseira tamariscifolia* for the first time. Here we show evidence that

111 exposure of hepatocarcinoma HepG2 cells to isololiolide is associated with changes in

112 the expression of p53, PARP, Bcl-2 and procaspase-3. These results might explain the

113 dramatic suppression of the S phase as well as the induction of apoptosis caused by this

114 monoterpene.

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116 **Material and methods**

117 **Chemicals and reagents**

118 Hexane and ethyl acetate were purchased from Prolabo (VWR International, Leuven,

119 Belgium). Merck (Darmstadt, Germany) supplied dimethyl sulfoxide (DMSO). Roswell

120 Park Memorial Institute medium (RPMI), Dulbecco's Modified Eagle's medium

121 (DMEM), fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin were

122 obtained from Lonza Ibérica (Barcelona, Spain). 3-(4,5-dimethylthiazol-2-yl)-2,5-

123 diphenyltetrazolium bromide (MTT) was obtained from Calbiochem. Primary antibodies

124 for poly (ADP-ribose) polymerase (PARP), p53, Bcl-2, actin and respective secondary

125 antibodies were from Santa Cruz Biotechnology Inc., Heidelberg, Germany. FITC-

126 conjugated annexin V/ propidium iodide (PI) assay kit was acquired from Cayman

127 Chemical Company, USA. Silica gel (Merck, 40-63 µm mesh) was used for column

128 chromatographic separation, while silica gel 60 PF_{254} (Merck) was used for analytical

129 (0.25 mm) TLC. $CDCl_3$ (Aldrich) was used as solvent for ^1H and ^{13}C NMR spectra

130 acquisition and TMS (Aldrich) was used as internal standard. 1D and 2D NMR spectra

were recorded at Bruker Digital Avance 800 MHz spectrometer. Additional reagents and
necessary solvents were purchased from VWR International (Leuven, Belgium).

Sampling

Cystoseira tamariscifolia was collected in the middle/lower intertidal areas, during the
low tide, between May and September 2012 on the Portuguese coast. Biomass was rinsed
with distilled water and macroscopic epiphytes and extraneous matter were carefully
removed. Identification of specimens was made by Dr Aschwin Engelen (Centre of
Marine Sciences, University of Algarve, Portugal) and Dr Javier Cremades Ugarte
(Facultade de Ciencias, University of A Coruña) and a voucher specimen of *C.
tamariscifolia* (code number MB016) was deposited at the Centre of Marine Sciences
(CCMAR), University of Algarve. Samples were freeze-dried and stored at -20 °C until
the extraction procedure.

Extraction

Biomass was mixed with hexane (1:10, w/v) and homogenized for 2 minutes using a
disperser IKA T10B Ultra-Turrax at room temperature (RT). The tubes were then
vortexed for 1 minute, centrifuged (5000 g, 10 minutes, RT) and the supernatants
recovered. The extraction procedure was repeated 3 times and the supernatants combined
and filtered. The extract was dried at 40 ℃ under vacuum and dissolved in DMSO for
biological activities screening or in the adequate solvent for chemical characterization,
aliquoted and stored (-20 °C).

Isolation and elucidation of isololiolide

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157 *C. tamariscifolia* hexane extract (9 g) was fractionated by column chromatography (2.5

158 cm × 18 cm) over silica gel (SiO_2) using increasing amounts of EtOAc in hexane (9:1;

159 85:15; 4:1; 75:25; 7:3; 3:2; 1:1) and increasing amounts of MeOH in EtOAc (9:1; 8:1;

160 5:1; 2:1; 1:1), MeOH (100%) and H_2O (100%) as eluents. This procedure afforded 57

161 fractions, which were analyzed by TLC and pooled together in 21 groups (A – U).

162 Fraction 14 (70 mg) was re-fractionated over SiO_2 eluted with hexane (100 %);

163 hexane/EtOAc (9:1, 8:2, 7.5:2.5, 7:3, 6.5:3.5, 6:4, 5.5:4.5, 1:1, 4:6), EtOAc (100 %) and

164 MeOH (100 %) to afford 151 fractions which were pooled together in 9 groups after TLC

165 analysis. Group 6 – 8, obtained from the hexane/ EtOAc elution (6:4 through 1:1), was

166 purified by reverse phase preparative HPLC to afford 3 mg of isololiolide.

167 Isololiolide. Pale yellow oil;^{1}H NMR (800 MHz, $CDCl_3$, TMS, ppm) δ 5.71 (1H, s, H-7),

168 4.21 (1H, m, H-3), 2.55 (2H, br d, J = 2.4 Hz, H-4), 2.03 (1H, br d, J = 2.4 Hz, H-2), 1.59

169 (3H, s, H-11), 1.23 (3H, s, H-10), 1.21 (3H, s, H-9). ^{13}C-NMR δ (200 MHz, $CDCl_3$, TMS,

170 ppm): 181.2 (C-6), 171.5 (C-8), 113.3 (C-7), 86.4 (C-5), 65.1 (C-3), 49.8 (C-2), 47.9 (C-

171 4), 35.0 (C-1), 29.9 (C-9), 25.6 (C-11), 25.1 (C-10); HRESIMS *m/z* 219.0993 [M + Na]$^+$

172 (calc to $C_{11}H_{16}O_3Na$ 219.0997).

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174 Cell culture

175 HepG2 cells (human hepatocellular carcinoma) were maintained in RPMI-1640 culture

176 media supplemented with glucose (1000 mg/ml), 10% FBS, L-glutamine (2 mM),

177 penicillin (50 U/ml) and streptomycin (50 µg/ml). MRC-5 and HFF-1 human fibroblasts,

178 AGS human gastric cancer, HCT-15 human colon cancer cells were grown in DMEM

179 culture media supplemented with glucose (1000 mg/ml), 10% FBS, L-glutamine (2 mM),

180 penicillin (50 U/ml) and streptomycin (50 µg/ml). Cell lines were grown in an incubator

181 at 37 ºC and 5.0% CO_2 in humidified atmosphere.

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Anti-proliferative assay

In vitro cytotoxic activity of isololiolide was assessed by the MTT colorimetric assay. Hepatocarcinoma HepG2, gastric cancer AGS and colon cancer HCT-15, and also non-tumoral cells (MRC-5 and HFF-1 human fibroblasts) were seeded at a density of 5×10^3 cells/well on 96-well plates and incubated for 24 h at 37 °C in 5.0% CO_2. The effect of isololiolide was evaluated on the viability of these cells and the half maximal inhibitory concentration (IC_{50}) was calculated upon a 72 h incubation period. Positive control cells were treated with etoposide, while negative control cells were treated with DMSO at the highest concentration used in test wells (0.5%, v/v). The selectivity of the compound was estimated using the following equation: Selectivity = CT/CNT, where CT and CNT indicate the compound-induced cytotoxicity on tumoral cells and on non-tumoral cells, respectively (Oh et al., 2010).

Cell cycle distribution analysis

HepG2 cells were plated at a density of 5×10^4 cells/ml in 6-well plates and incubated with complete medium only (blank), medium with the solvent DMSO (control, 0.5% v/v) or with isololiolide at IC_{50} concentration (13.15 μM), which was previously determined by the MTT assay. Cells were harvested following 72 h incubation and further processed for cell cycle analysis. Cellular DNA content for cell cycle distribution analysis was evaluated using an Epics XL-MCL Coulter flow cytometer plotting at least 10000 events per sample. Cell cycle distribution data analysis was subsequently performed using the FlowJo 7.2 software (Tree Star, Ashland, USA).

Apoptosis detection

HepG2 cells were plated at a concentration of 5×10^4 cells/ml in 6-well plates and incubated with complete medium only (blank), medium with the solvent DMSO (control, 0.5% v/v), or with isololiolide at IC_{50} concentration (13.15 µM) for 72 h. Induction of apoptosis was evaluated by the annexin V-FITC/PI apoptosis Kit (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions. Measurement of annexin V binding due to phosphatidylserine externalization was analyzed using an Epics XL-MCL Coulter flow cytometer plotting at least 20 000 events per sample. Apoptotic data analysis was subsequently performed using the FlowJo 7.2 software (Tree Star, Ashland, USA).

Protein expression analysis

For the analysis of protein expression, HepG2 cells were treated with complete medium (blank), medium with the solvent (DMSO) or with loliolide at the IC_{50} concentration (13.15 µM), and incubated for 24, 48 and 72 h. After each incubation period cells were lysed in Winman's buffer (1% NP-40, 0.1 M Tris–HCl pH 8.0, 0.15 M NaCl and 5 mM EDTA) with EDTA-free protease inhibitor cocktail (Boehringer, Mannheim, Germany). Proteins were quantified using the DC Protein Assay Kit (BioRad, Hercules, CA, USA) and separated in 12% tris-glycine sodium dodecyl sulphate (SDS)–polyacrylamide gel. Proteins were then transferred to a nitro-cellulose membrane (GE Healthcare, Madrid, Spain). The membranes were incubated with the following primary antibodies for PARP (1:4000), actin (1:2000), p53 (1:250), Bcl-2 (1:200) and procaspase-3 (1:2000), and further incubated with the appropriate secondary antibodies conjugated with horseradish peroxidase (HRP) diluted 1:2000 in 5% non-fat dried milk in T-TBS. The signal was

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232 detected with the Amersham ECL kit (GE Healthcare). Hyperfilm ECL (GE Healthcare)

233 and Kodak GBX developer and fixer twin pack (Sigma).

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235 **Statistical analysis**

236 Results were expressed as mean ± standard error of the mean (SEM). Analysis of variance

237 (ANOVA) was assessed using the SPSS statistical package for Windows (release 15.0,

238 SPSS Inc.), and significance between means was analyzed by the Tukey HSD test ($p <$

239 0.05). IC_{50} values were calculated by sigmoidal fitting of the data using GraphPad Prism

240 v. 5.0 (GraphPad Software, Inc., La Jolla, CA). Statistical analysis was performed by the

241 non-parametric Friedman's test followed by Dunn's Post-test using GraphPad Prism 5

242 software. *P* values < 0.05 were considered as statistically significant.

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244 **Results and discussion**

245 **Characterization of isololiolide**

246 HRESIMS of the isolated compound showed a $[M + Na]^+$ *quasi*-molecular ion peak at

247 *m/z* 219.0993, indicating the molecular formula $C_{11}H_{16}O_3$, with four unsaturations. Its ^{1}H

248 NMR spectrum displayed, despite other signals, peaks assigned to hydrogens of three

249 methyl groups at δ_H 1.21 (s, 3H), 1.23 (s, 3H) and 1.59 (s, 3H), one olefinic hydrogen at

250 δ_H 5.71 (s, 1H) and one oxymethine hydrogen at δ_H 4.13 (m, 1H). The ^{13}C and DEPT 135

251 showed eleven peaks assigned to three methyl, two methylene, two methine and four

252 quaternary carbons, including one α,β-unsaturated carbonyl group at δ_C 171.5 (C-8),

253 113.3 (C-7) and 181.2 (C-8) and one carbinolic carbon at δ_C 65.1 (C-3). HMBC spectrum

254 showed cross peaks between the signals at H-11 with C-4/C-5/C-6, H-9 with C-1/C-6/C-

255 10, H-10 with C-2/C-6/C-9 and H-7 with C-5/C-6/C-8. Isololiolide (Fig. 1) was identified

256 comparing the obtained data with that reported in the literature (Kimura and Maki 2002).

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Anti-proliferative activity of isololiolide in tumoral and non-tumoral cell lines

Isololiolide obtained from the *C. tamariscifolia* hexane extract was tested on human hepatocellular carcinoma cells (HepG2), gastric cancer cells (AGS) and colon cancer cell line (HCT-15). Additionally, the anti-proliferative activity of the compounds was evaluated in human fibroblasts (MRC-5 and HFF-1). The compound proved to be cytotoxic against the different tumoral cell lines, namely AGS (IC_{50} = 32.36 µM), HCT-15 (IC_{50} = 23.59 µM) and especially HepG2 cells (IC_{50} = 13.15 µM; Table 1), showing selectivity indices (SI) of up to 86 and 47 against MRC-5 and HFF-1 fibroblasts, respectively (Table 1). The cytotoxic effect of this molecule towards HepG2 cells was particularly evident, whereas no significant toxic effect was observed in MRC-5 or HFF-1 human fibroblasts. Interestingly, extracts from *C. tamariscifolia* had previously demonstrated antiproliferative potential against Daudi (human Burkitt's lymphoma), Jurkat (human leukemic T cell lymphoblast) and K562 (human chronic myelogenous leukemia) cells (Zubia et al. 2009). Isololiolide has been described as a carotenoid metabolite whose precursor is zeaxanthin (Repeta 1989). Carotenoids, such as zeaxanthin, lycopene and astaxanthin have been previously described as modulators of growth factors that play important roles in cell cycle regulation and carcinogenesis (Bi et al. 2013, Alvarez et al. 2014). Moreover, it has been previously demonstrated that breakdown products of carotenoids (e.g. 3-OH-β-apo-10'-carotenal and apo-10'-lycopenal) might act as chemotherapeutic agents against breast and hepatic cancer (Tibaduiza et al. 2002, Ip et al. 2014). Loliolide, an isololiolide isomer, is also a well-known carotenoid metabolite derived from the breakdown from fucoxanthin able to inhibit algal growth (Taylor and Burden 1970). On the other hand, isololiolide has been previously isolated from brown algae namely from *Undaria pinnatifida* (Kimura and

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282 Maki 2002), *Dictyopteris divaricata* (Song et al. 2004) and *Homoeostrichus formosana*

283 (Fang et al. 2015). However, this is the first report describing the occurrence of

284 isololiolide in *C. tamariscifolia*.

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286 **Effects on cell cycle profile**

287 Because of the potential application of carotenoid breakdown products in cancer

288 therapeutics and the observed cytoxicity in HepG2 cells, we researched the effectiveness

289 of isololiolide in arresting the cell cycle in the latter hepatocarcinoma cell line. For this

290 purpose, HepG2 cells were incubated with isololiolide at 13.15 µM (IC$_{50}$) for 72 h and its

291 effect on cell cycle distribution was studied. Analysis of the cell cycle was performed by

292 flow cytometry and the results showed that this monoterpenoid completely disrupted the

293 normal HepG2 cell cycle. In fact, isololiolide induced G2/M cell cycle arrest along with

294 a concomitant decrease in the percentage of cells in the S phase (Fig. 2AA) and this effect

295 was sustained throughout the 72 h treatment. In fact, the percentage of cells in G2/M

296 phase was 15.09 % and 14.91 % for the control and DMSO 0.5 %, respectively, increasing

297 to 57.95 % upon treatment with isololiolide at 13.15 µM for 72 h (Fig. 2BB). In addition,

298 there were virtually no cells in the S phase after the same incubation. Taken together,

299 these results suggest that isololiolide affects the molecular pathways monitoring and

300 controlling cell cycle progression by arresting the cells at the G2/M checkpoint. The cell

301 cycle checkpoints play an important role in the control system by sensing defects

302 occurring during essential processes, such as DNA replication or chromosome

303 segregation, inducing a cell cycle arrest until the defects detected are repaired (Malumbres

304 2012).

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306 **Apoptosis induction by isololiode treatment**

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The annexin V-FITC/PI flow cytometry assay was used in order to determine if isololiolide was inducing apoptosis in HepG2 cells. Bivariate staining using annexin V-FITC/PI further demonstrated that isololiolide induced apoptosis at the IC_{50} concentration (Fig. 3AA). HepG2 cells were treated with isololiolide for 72 h and a significant increase ($P < 0.01$) in the percentage of apoptotic cells was observed, from 6.9 % in untreated cells, to 29.1 % in cells treated with isololiolide (Fig. 3BB). Apoptosis is described as an active process of programmed cellular death that avoids an exacerbated inflammatory response (Fink and Cookson 2005) and is associated with responses to cancer therapy. In fact, it is widely described that resistance to apoptosis is one of the hallmarks of cancer cells (Hanahan and Weinberg 2011). This resistance enables cancerous cells to survive and divide even in the presence of endogenous proapoptotic stimuli. Therefore, induction of apoptosis is an important mechanism in selecting novel molecules with anti-cancer potential.

Western blot analysis of apoptosis-related proteins

Based on the results obtained with annexin V-FITC demonstrating that apoptosis is occurring, the expression of apoptosis-related proteins was evaluated by assessing procaspase-3, PARP, Bcl-2 and p53 protein levels expression in HepG2 cells incubated with complete medium (control), vehicle (DMSO 0.5% v/v) or isololiolide (13.15 µM). Concerning caspase-3, a decrease in procaspase-3 expression upon isololiolide treatment at 24 h was measured (Fig. 4, $P < 0.05$). In human cells, apoptosis takes place through a cascade of events involving two main pathways: the intrinsic and the extrinsic pathways (Kroemer et al. 2007). Both pathways ultimately converge on the activation of procaspases (primarily procaspase-3, but also procaspase-7 and procaspase-6) to caspases, which are the cysteine proteases that cleave their protein substrates within the

332 cell. Effector caspase-3 zymogen (or procaspase-3) exists within the cytosol as an inactive

333 dimer (Boatright and Salvesen 2003). It is activated by limited proteolysis within the

334 interdomain linker, which is carried out by an initiator caspase or occasionally by other

335 proteases under specific circumstances. At cytosolic concentrations in human cells, the

336 caspase-3 zymogens are already dimers, but cleavage within their respective linker

337 segments is required for activation (Boatright and Salvesen 2003). Therefore, a decrease

338 in procaspase-3 levels is due to its proteolysis, leading to caspase-3 activation. Our results

339 showed that incubation of HepG2 cells with isololiolide resulted in a 2-fold decrease of

340 procaspase-3 levels, strongly suggesting that procaspase-3 was processed to caspase-3.

341 In addition, concentrations of procaspase-3 in certain cancerous cells are significantly

342 higher than those in non-cancerous controls (Putt et al. 2006).

343 PARP cleavage increased about 4-fold upon isololiolide incubation at 13.15 µM for 24

344 h (Fig. 5, $P < 0.01$). Interestingly, this increment seems to be time-dependent as shown in

345 Fig. 5. PARP plays an active role in key biological processes, such as transcription and

346 cell cycle regulation, response to DNA damage, apoptosis and maintenance of genome

347 integrity. The presence of cleaved PARP is one of the most used biomarkers for the

348 detection of apoptosis (Duriez and Shah 1997). Moreover, PARP is a substrate of caspase-

349 3 and its cleavage into two fragments has been considered to be indicative of functional

350 caspase activation (Bressenot et al. 2009). Cleaved PARP was observed after treatment

351 with isololiolide, in a time-dependent manner. In fact, (Soldani et al. 2001) reported that

352 PARP proteolysis by caspase is a very early response to the apoptotic stimulus.

353 Western blot performed in the cell lysates obtained from isololiolide-treated cells showed

354 increased expression of p53 at 24 h (Fig. 6, $P < 0.05$ *vs.* DMSO 0.5 %). The tumor

355 suppressor protein p53 acts as a key player in tumor suppression, as it induces apoptosis

356 and cell cycle arrest as well as suppress angiogenesis (Amaral et al. 2010). p53 is usually

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357 responsible for activating DNA repair proteins when DNA has extensive damage,

358 arresting the cell cycle at regulation points or initiating apoptosis if DNA damage shows

359 to be irreparable. Interestingly, p53 not only induces G1 cell cycle arrest, but it is also

360 described to act at the G2/M checkpoint, preventing cells from entering mitosis if DNA

361 damage is found (Taylor and Stark 2001). Furthermore, p53 has the ability to activate the

362 transcription of various pro-apoptotic genes, including those encoding members of the

363 Bcl-2 family (Roos and Kaina 2006).

364 Our results showed that anti-apoptotic Bcl-2 protein expression remained unchanged after

365 24 h of incubation with isololiolide, decreasing after 48 h with the same treatment (Fig.

366 7, $P < 0.05$ *vs* DMSO 0.5 %). Overexpression of anti-apoptotic Bcl-2 family members

367 have been associated with chemotherapy resistance in various human cancers, and

368 targeting the anti-apoptotic Bcl-2 family members have shown promising results in

369 preclinical studies (Kang and Reynolds 2009). Bcl-2 suppresses apoptosis by binding to

370 Bax or Bak. It is described that inhibiting the anti-apoptotic Bcl-2 could sensitize tumor

371 cells to chemo- and radiotherapy. Therefore, decrease of Bcl-2 levels may be a plan of

372 choice to increase treatment efficacy. Furthermore, it was suggested that p53 also

373 modulates Bcl-2 by downregulation (Kirkin et al. 2004).

374 In summary, application of isololiode resulted in the increase on caspase-3 expression,

375 concomitant with increase in PARP cleavage and p53 expression. Corresponding down-

376 regulation of anti-apoptotic/pro-survival Bcl-2 protein was also detected.

377 Indeed, molecules that activate caspase-3 and p53, cleave PARP or bind to Bcl-2 have

378 shown potential in cell culture and preclinical models of cancer (Peterson et al. 2009).

379 Taken together, our results strongly suggest that isololiolide is able to exert potent anti-

380 proliferative properties, significantly promoting cell cycle arrest in S phase and inducing

381 cellular apoptosis in a human-derived hepatocarcinoma cell line.

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Conclusions

This report demonstrates for the first time the *in vitro* anti-tumoral activity of isololiolide obtained from *C. tamariscifolia* hexane extract against hepatocarcinoma through the induction of apoptosis by altering the expression of proteins important to the apoptotic cascade. As isololiolide exhibited no cytotoxicity on non-tumoral human fibroblasts under the same conditions, it would be important in the future to perform structure-activity relationships (SARs) analysis for further studies. In addition, it may provide novel clues as to how carotenoids and their metabolites play a role in preventing and/or slowing down cancer progression.

Conflicts of interest

The authors declare no conflict of interest.

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Figure legends

Fig. 1. Chemical structure of isololiolide.

Fig. 2. HepG2 cell cycle analysis (A) from control (incubation with complete medium only), DMSO (0.5% v/v) or isololiolide (13.15 µM) treatment for 72 h. Percentage of cells in G1, S and G2/M phases upon the treatments described above (B).

Fig. 3. Flow cytometric analysis (A) and the proportion of apoptotic (B) HepG2 cells treated for 72 h with control (incubation with complete medium only), DMSO (0.5% v/v) or isololiolide (13.15 µM) for 72 h stained for annexin V-FITC/PI. **$P < 0.01$ *vs.* DMSO 0.5 %.

Fig. 4. Procaspase-3 expression levels upon incubation with complete medium (control), vehicle (DMSO 0.5 %) or isololiolide (13.15 µM) at 24 h. *$P < 0.05$ *vs.* DMSO 0.5 %.

Fig. 5. Full length (PARP) and cleaved PARP (PARP*) expression levels upon incubation with complete medium (control), vehicle (DMSO 0.5 %) or isololiolide (13.15 µM) at 24, 48 and 72 h. *$P < 0.05$, **$P < 0.01$ *vs.* DMSO 0.5 %.

Fig. 6. p53 expression levels upon incubation with complete medium (control), vehicle (DMSO 0.5 %) or isololiolide (13.15 µM) at 24 h.*$P < 0.05$ *vs.* DMSO 0.5 %.

Fig. 7. Bcl-2 expression levels upon incubation with complete medium (control), vehicle (DMSO 0.5 %) or isololiolide (13.15 µM) at 24 and 48 h. *$P < 0.05$ *vs.* DMSO 0.5 %.