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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6 Catarina Vizetto-Duarte[a], Luísa Custódio[a], Katkam N. Gangadhar[a], João Henrique G. 7 Lago[b], Catarina Dias[c], Ana Marta Matos[c], Nuno Neng[c], José Manuel Florêncio Nogueira[c], 8 Luísa Barreira[a], Fernando Albericio[d,e,f], Amélia P. Rauter[c] and João Varela[a,*]

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10 [a]Centre of Marine Sciences, University of Algarve, Faculty of Sciences and Technology, 11 Ed. 7, Campus of Gambelas, Faro, Portugal

12 [b]Institute of Environmental, Chemical and Pharmaceutical Sciences, Federal University 13 of Sao Paulo, 09972-270, Sao Paulo, Brazil

14 [c]Center of Chemistry and Biochemistry, Department of Chemistry and Biochemistry, 15 Faculty of Sciences University of Lisbon, Campo Grande, Ed. C8, Piso 5, 1749-016 16 Lisbon, Portugal

17 [d]Institute for Research in Biomedicine, Barcelona Science Park, Baldiri Reixac 10, 18 08028, Barcelona, Spain,

19 [e]CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, 20 Barcelona Science Park, Baldiri Reixac 10, 08028 Barcelona, Spain,

21 [f]University of Barcelona, Department of Organic Chemistry, Martí i Franqués 1-11, 22 08028 Barcelona, Spain.

23

24

25 **Corresponding author.*

26 Centre of Marine Sciences, University of Algarve, Faculty of Sciences and Technology, 27 Ed. 7, Campus of Gambelas, 8005-139 Faro, Portugal.

28 Tel.:+351 289 800051; fax: +351 289800051.

29 *E-mail address:* jvarela@ualg.pt

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32 **ABSTRACT**

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

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

37 *Methods/Study design:* The structure of the compound was established and confirmed by
38 1D and 2D NMR as well as HRMS spectral analysis. The *in vitro* cytotoxicity was
39 analyzed by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
40 assay in tumoral as well as in non-tumoral cell lines. Cell cycle arrest and induction of
41 apoptosis were assessed by flow cytometry. Alteration of expression levels in proteins
42 important in the apoptotic cascade was analyzed by western blotting.

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

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

54

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

Abbreviations: 1D and 2D NMR, one-dimensional and two-dimensional nuclear magnetic resonance; ^{1}H and ^{13}C NMR, proton and carbon-13 nuclear magnetic resonance; ANOVA, analysis of variance; Bak, Bcl-2 homologous antagonist killer; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; $CDCl_3$, deuterated chloroform; CNT, compound-induced cytotoxicity on non-tumoral cells; CT, compound-induced cytotoxicity on tumoral cells; DEPT, distortionless enhancement by polarization transfer; DMEM, Dulbecco's Modified Eagle's medium; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; EtOAc, ethyl acetate; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HCC, hepatocellular carcinoma; HPLC, high performance liquid chromatography; HRESIMS, high-resolution electrospray ionisation mass spectrometry; HRMS, high resolution mass spectrometry; HRP, horseradish peroxidase; HSD, honest significant difference; IC_{50}, half maximal inhibitory concentration; *m/z*, mass-to-charge ratio; MeOH, methanol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NP-40, nonidet P-40; p53, protein 53; PARP, poly (ADP-ribose) polymerase; PI, propidium iodide; RPMI, Roswell Park Memorial Institute medium; SARs, structure-activity relationships; SDS, sodium dodecyl sulphate; SEM, standard error of mean; SiO_2, silica; TLC, thin layer chromatography; TMS, tetramethylsilane; WHO, World Health Organization.

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,

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 ^{1}H and ^{13}C NMR spectra
130 acquisition and TMS (Aldrich) was used as internal standard. 1D and 2D NMR spectra

131 were recorded at Bruker Digital Avance 800 MHz spectrometer. Additional reagents and
132 necessary solvents were purchased from VWR International (Leuven, Belgium).
133

134 **Sampling**

135 *Cystoseira tamariscifolia* was collected in the middle/lower intertidal areas, during the
136 low tide, between May and September 2012 on the Portuguese coast. Biomass was rinsed
137 with distilled water and macroscopic epiphytes and extraneous matter were carefully
138 removed. Identification of specimens was made by Dr Aschwin Engelen (Centre of
139 Marine Sciences, University of Algarve, Portugal) and Dr Javier Cremades Ugarte
140 (Facultade de Ciencias, University of A Coruña) and a voucher specimen of *C.*
141 *tamariscifolia* (code number MB016) was deposited at the Centre of Marine Sciences
142 (CCMAR), University of Algarve. Samples were freeze-dried and stored at -20 °C until
143 the extraction procedure.
144

145 **Extraction**

146 Biomass was mixed with hexane (1:10, w/v) and homogenized for 2 minutes using a
147 disperser IKA T10B Ultra-Turrax at room temperature (RT). The tubes were then
148 vortexed for 1 minute, centrifuged (5000 *g*, 10 minutes, RT) and the supernatants
149 recovered. The extraction procedure was repeated 3 times and the supernatants combined
150 and filtered. The extract was dried at 40 ºC under vacuum and dissolved in DMSO for
151 biological activities screening or in the adequate solvent for chemical characterization,
152 aliquoted and stored (-20 °C).
153
154
155

156 **Isolation and elucidation of isololiolide**

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).
173
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.
182

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).

208 **Apoptosis detection**

209 HepG2 cells were plated at a concentration of 5×10^4 cells/ml in 6-well plates and
210 incubated with complete medium only (blank), medium with the solvent DMSO (control,
211 0.5% v/v), or with isololiolide at IC_{50} concentration (13.15 μM) for 72 h. Induction of
212 apoptosis was evaluated by the annexin V-FITC/PI apoptosis Kit (Bender MedSystems,
213 Vienna, Austria) according to the manufacturer's instructions. Measurement of annexin
214 V binding due to phosphatidylserine externalization was analyzed using an Epics XL-
215 MCL Coulter flow cytometer plotting at least 20 000 events per sample. Apoptotic data
216 analysis was subsequently performed using the FlowJo 7.2 software (Tree Star, Ashland,
217 USA).

218

219 **Protein expression analysis**

220 For the analysis of protein expression, HepG2 cells were treated with complete medium
221 (blank), medium with the solvent (DMSO) or with loliolide at the IC_{50} concentration
222 (13.15 μM), and incubated for 24, 48 and 72 h. After each incubation period cells were
223 lysed in Winman's buffer (1% NP-40, 0.1 M Tris–HCl pH 8.0, 0.15 M NaCl and 5 mM
224 EDTA) with EDTA-free protease inhibitor cocktail (Boehringer, Mannheim, Germany).
225 Proteins were quantified using the DC Protein Assay Kit (BioRad, Hercules, CA, USA)
226 and separated in 12% tris-glycine sodium dodecyl sulphate (SDS)–polyacrylamide gel.
227 Proteins were then transferred to a nitro-cellulose membrane (GE Healthcare, Madrid,
228 Spain). The membranes were incubated with the following primary antibodies for PARP
229 (1:4000), actin (1:2000), p53 (1:250), Bcl-2 (1:200) and procaspase-3 (1:2000), and
230 further incubated with the appropriate secondary antibodies conjugated with horseradish
231 peroxidase (HRP) diluted 1:2000 in 5% non-fat dried milk in T-TBS. The signal was

232 detected with the Amersham ECL kit (GE Healthcare). Hyperfilm ECL (GE Healthcare)
233 and Kodak GBX developer and fixer twin pack (Sigma).
234

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.
243

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 ^1H
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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258 **Anti-proliferative activity of isololiolide in tumoral and non-tumoral cell lines**

259 Isololiolide obtained from the *C. tamariscifolia* hexane extract was tested on human
260 hepatocellular carcinoma cells (HepG2), gastric cancer cells (AGS) and colon cancer cell
261 line (HCT-15). Additionally, the anti-proliferative activity of the compounds was
262 evaluated in human fibroblasts (MRC-5 and HFF-1). The compound proved to be
263 cytotoxic against the different tumoral cell lines, namely AGS (IC_{50} = 32.36 µM), HCT-
264 15 (IC_{50} = 23.59 µM) and especially HepG2 cells (IC_{50} = 13.15 µM; Table 1), showing
265 selectivity indices (SI) of up to 86 and 47 against MRC-5 and HFF-1 fibroblasts,
266 respectively (Table 1). The cytotoxic effect of this molecule towards HepG2 cells was
267 particularly evident, whereas no significant toxic effect was observed in MRC-5 or HFF-
268 1 human fibroblasts. Interestingly, extracts from *C. tamariscifolia* had previously
269 demonstrated antiproliferative potential against Daudi (human Burkitt's lymphoma),
270 Jurkat (human leukemic T cell lymphoblast) and K562 (human chronic myelogenous
271 leukemia) cells (Zubia et al. 2009). Isololiolide has been described as a carotenoid
272 metabolite whose precursor is zeaxanthin (Repeta 1989). Carotenoids, such as
273 zeaxanthin, lycopene and astaxanthin have been previously described as modulators of
274 growth factors that play important roles in cell cycle regulation and carcinogenesis (Bi et
275 al. 2013, Alvarez et al. 2014). Moreover, it has been previously demonstrated that
276 breakdown products of carotenoids (e.g. 3-OH-β-apo-10'-carotenal and apo-10'-
277 lycopenal) might act as chemotherapeutic agents against breast and hepatic cancer
278 (Tibaduiza et al. 2002, Ip et al. 2014). Loliolide, an isololiolide isomer, is also a well-
279 known carotenoid metabolite derived from the breakdown from fucoxanthin able to
280 inhibit algal growth (Taylor and Burden 1970). On the other hand, isololiolide has been
281 previously isolated from brown algae namely from *Undaria pinnatifida* (Kimura and

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Maki 2002), *Dictyopteris divaricata* (Song et al. 2004) and *Homoeostrichus formosana* (Fang et al. 2015). However, this is the first report describing the occurrence of isololiolide in *C. tamariscifolia*.

Effects on cell cycle profile

Because of the potential application of carotenoid breakdown products in cancer therapeutics and the observed cytoxicity in HepG2 cells, we researched the effectiveness of isololiolide in arresting the cell cycle in the latter hepatocarcinoma cell line. For this purpose, HepG2 cells were incubated with isololiolide at 13.15 µM (IC_{50}) for 72 h and its effect on cell cycle distribution was studied. Analysis of the cell cycle was performed by flow cytometry and the results showed that this monoterpenoid completely disrupted the normal HepG2 cell cycle. In fact, isololiolide induced G2/M cell cycle arrest along with a concomitant decrease in the percentage of cells in the S phase (Fig. 2A~~A~~) and this effect was sustained throughout the 72 h treatment. In fact, the percentage of cells in G2/M phase was 15.09 % and 14.91 % for the control and DMSO 0.5 %, respectively, increasing to 57.95 % upon treatment with isololiolide at 13.15 µM for 72 h (Fig. 2B~~B~~). In addition, there were virtually no cells in the S phase after the same incubation. Taken together, these results suggest that isololiolide affects the molecular pathways monitoring and controlling cell cycle progression by arresting the cells at the G2/M checkpoint. The cell cycle checkpoints play an important role in the control system by sensing defects occurring during essential processes, such as DNA replication or chromosome segregation, inducing a cell cycle arrest until the defects detected are repaired (Malumbres 2012).

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Apoptosis induction by isololiode treatment

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. 3A). 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. 3B). 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

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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).

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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.

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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).

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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).

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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).

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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.

382

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.

392

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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 %.