Constituents of *Carpobrotus edulis* Inhibit P-Glycoprotein of *MDR1*-transfected Mouse Lymphoma Cells

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Abstract. *A bioassay-guided separation protocol, including the testing of the extracts, fractions and pure compounds for their ability to inhibit P-glycoprotein (the efflux pump responsible for the multidrug resistance of the used cell line) of mouse lymphoma cells containing the human efflux pump gene MDR1, led to the isolation of seven compounds from the chloroform and ethyl acetate soluble fractions of the methanolic extract of Carpobrotus edulis. The compounds were identified by 1D, 2D NMR and MS investigations as triterpens (β-amyrin, uvaol and oleanolic acid), monogalactosyldiacylglycerol, catechin, epicatechin and procyanidin B5. Uvaol was the most effective and promising compound in the reversal of multidrug resistance in MDR mouse lymphoma cell line.*

Multidrug resistance (MDR) of cancer and to bacterial infections caused by *Staphylococcus aureus* or *Mycobacterium tuberculosis* are major health problems that affect therapy. The use of compounds that reverse resistance of MDR pathologies is now a widely accepted approach for the therapy of multidrug resistance (1-3).

MDR of cancer is mainly due to efflux pumps that are overexpressed as a consequence of treatment. These efflux pumps extrude the anticancer drug before it can reach its intended target (4). Moreover, for reasons that remain unclear, these efflux systems not only extrude the drug to which the cancer has become refractory, but also many other compounds used for chemotherapy of cancer (5), hence, their MDR phenotype. It should be noted some untreated tumors are also of an MDR phenotype which results from an overexpressed intrinsic efflux pump (6). Among the 12 or so transporter proteins that make up such efflux pumps of MDR is P-glycoprotein (P-gp), an ATP-binding cassette (ABC) transporter that is coded by the gene *MDR1* and which may be overexpressed intrinsically or as a consequence of chemotherapy (7). The energy of the ABC type transporters needed for their activity is derived from the hydrolysis of ATP (8). Although the molecular mechanism for the extrusion of a wide range of structurally diverse drugs remains unclear, the extrusion of drugs by P-gp can be blocked by many compounds, such as nifedipine, verapamil, quinine, chloroquine, progestogens, tamoxifen, cyclosporin A and its analogues, reserpine and tricyclic antidepressants (9, 10). However, most of these compounds are toxic at concentrations that block the P-gp pump. As an example, verapamil at a concentration of 2 mg/L blocks the P-gp pump but also produces congestive cardiac failure (11).

Plants are rich sources of new drugs for the therapy of cancer (12-14) and bacterial infections (2, 15-17). Some of the compounds derived from plants can serve as adjuvants to current chemotherapy inasmuch as they reverse MDR of cancer (18) and MDR of bacterial infections (17). One of these plants is *Carpobrotus edulis*; its methanolic extract has been shown to have activity against MDR cancer and MDR *M. tuberculosis* (19, 20). This succulent plant is very common in sandy and dune areas of Portugal and other Mediterranean countries (21, 22), coastal California (23) and South Africa. In the latter country, *C. edulis* is used in traditional medicine for the treatment of burns, stomach ailments, ulcerations of the mouth, toothache, oral and vaginal thrush, as well as bacterial infections, such as those affecting the lungs (tuberculosis), sinuses, and the colon (dysentery) (22, 24-27).

Previously, Ordway *et al.* showed that the methanolic extract of *C. edulis* reverses resistance of mouse lymphoma cells carying the human *MDR1* gene to chemotherapeutic

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agents and increases the killing activity of *S. aureus*-infected macrophages (20). Martins *et al*. demonstrated that the same extract was active against MDR *M. tuberculosis* and methicillin-resistant *S. aureus* (19). Other researchers have shown that extracts of other *Carpobrotus* species are active against various bacteria (26). Compounds active against *Bacillus subtilis*, *Staphylococcus epidermis*, *S. aureus*, *Streptococcus pneumonia*, *Moraxella catarrhalis* and *Pseudomonas aeruginosa* have been isolated from *C. edulis* by Watt *et al*. (22). Among the identified active compounds were ferulic acid, flavonoids (rutin, hyperoside and neohesperidin) and cactichin (22).

Although the above studies were primarily restricted to activities of the extracts and compounds against bacteria, the observation of Ordway *et al*. that the methanolic extract demonstrated activity against MDR of cancer cells (20) spurred us to isolate and identify the active constituents of the methanolic extract of *C. edulis* responsible for the anti-MDR activity.

Materials and Methods

Plant material. The leaves of *C. edulis* (L.) N. E. Br. (Aizoaceae) were collected in November 2007 at Guincho (Sintra, Portugal). The plant was authenticated by Professor António Viveiros (Professor Emeritus of Botany, Plant Biology Department, Sciences Faculty of the University of Lisbon).

Cell cultures. Parental and MDR cell lines used in the biological assays were L5178 mouse T-cell lymphoma cells (ECACC cat. no. 87111908, U.S. FDA, Silver Spring, MD, USA) and the L5178 mouse T-cell lymphoma cells transfected with pHa MDR1/A retrovirus, as previously described (28). MDR cell lines were selected by culturing the infected cells with 60 ng/ml of colchicine to maintain the expression of the MDR phenotype (29). Both cell lines were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine and antibiotics (penicillin and streptomycin), at 37˚C and 5% CO_2 atmosphere. Medium, horse serum and antibiotics purchased from Difco, USA.

General purification procedures. For the purification, vacuum-liquid chromatography (VLC) and rotation planar chromatography (RPC) on polyamide (ICN) and on silica gel (Kieselgel GF_{254} 15 μm; Merck, Germany) were used. Gel chromatography was carried out on Sephadex LH-20. Preparative thin layer chromatography (TLC) was performed on silica gel plates (Merck 5715; Merck). High performance liquid chromatography (HPLC) was carried out on a Waters Millipore instrument (Millipore, Waters Chromatography Division, Milliford, USA), on a LiChrospher® 100 RP-18 (10 μm) column with MeOH-H_2O (3:7) as mobile phase with detection at 220 and 254 nm. Chromatographic fractions were monitored by normal phase (NP)-TLC on silica gel plates (60 F_{254}; Merck) and reversed phase (RP)-TLC (RP-18 F_{254S}, Merck), visualized by spraying with concentrated H_2SO_4, followed by heating.

Structural nuclear magnetic resonance (NMR) elucidation was carried out by extensive spectroscopic analysis, including 1D and 2D (^{1}H-^{1}H correlation spectroscopy (COSY), Heteronuclear Single Quantum Coherence (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC)) on a Bruker Avance DRX 500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C) (Bruker, Zurich, Switzerland). The stereochemistry of the compounds was studied by Nuclear Overhauser Effect Spectroscopy (NOESY) measurements. 1D NMR spectra were recorded. Two dimensional experiments were performed using the standard Bruker software.

Extraction. The fresh plant material of *C. edulis* (5 kg) was extracted with MeOH (13 l) at room temperature. The crude extract was concentrated under vacuum (Rotavapor-RE, Büchi) to 500 ml and extracted with n-hexane (1600 ml). The *n*-hexane was evaporated off leading fraction A. The aqueous methanolic phase was then extracted with chloroform (2.5 l). On evaporation, an organic phase residue was obtained (fraction B). Thereafter the methanol-water phase was extracted with ethyl acetate (1.2 l) which yielded a dry residue after evaporation (fraction C).

Isolation of the compounds. Fractions A and B were jointly chromatographed on a polyamide column with mixtures of H_2O–MeOH (4:1, 7:3, 3:2, 1:1, 2:3, 3:7 and 1:4) as eluents. The fractions obtained with 60, 70 and 80% MeOH elutions were combined and chromatographed on silica gel, using a gradient system of *n*-hexane–$CHCl_3$–MeOH (7:3:0, 3:2:0, 1:1:0, and 50:50:1), and $CHCl_3$–MeOH–H_2O (49:1:0, 9:1:0, 4:1:0 and 35:13:2). The fractions obtained with *n*-hexane–$CHCl_3$–MeOH (50:50:1) and $CHCl_3$–MeOH (49:1) were further purified on Sephadex LH-20 using MeOH as eluent. Fractions were then separated by using RPC on silica gel with *n*-hexane–CH_2Cl_2 (50:50) and increasing concentrations of MeOH (0.25, 0.5, 1, 2, 3, 5, 10 and 50, respectively) to yield compounds **1** (13.1 mg), **2** (14.3 mg) and **3** (12.4 mg) (Figue 1).

The fraction obtained from polyamide column with 50% MeOH was chromatographed on a silica gel column, described previously, using the same parameters. The fraction obtained with $CHCl_3$–MeOH (49:1) was further purified by preparative TLC with cyclohexane–EtOAc–EtOH (30:20:2) to obtain compound **4** (10.6 mg).

The ethyl acetate fraction (fraction C) was chromatographed on a silica gel (40 g) column with a gradient system of CH_2Cl_2–MeOH–H_2O (49:1:0, 9:1:0, 85:15:0, 4:1:0, 7:3:0 and 70:30:1), and MeOH:H_2O (4:1). Fractions eluted with CH_2Cl_2–MeOH (85:15 and 4:1) were separated on a silica gel column using a gradient of $CHCl_3$–MeOH (49:1 to 7:3) and the fraction eluted with $CHCl_3$–MeOH (4:1) was further purified by RP-HPLC. As a result of this separation, compounds **5** (7.7 mg) and **6** (5.6 mg) were obtained. The fraction eluted from silica gel column with CH_2Cl_2–MeOH–H_2O (70:30:1) was chromatographed on a Sephadex LH-20 column, then on preparative TLC plates with EtOAc–HCOOH–H_2O (49:2:1; NP-TLC), and then with MeOH:H_2O (1:1, RP-TLC) as developing systems, to yield compounds **5** and **6** and with AcNi:H_2O: CH_3COOH (30:70:0.5, RP-TLC) to yield compound **7** (3.6 mg).

Antiproliferative assay. The antiproliferative activity of the compounds against parental mouse lymphoma cells and their *MDR1* transfected progeny was tested in triplicate by the MTT microplate assay previously described in detail (30).

Checker-board assay. The compounds were evaluated by the checkerboard microplate method for the study of drug interaction between the pure compound and doxorubicin on MDR cancer cells. This method is similar to the antiproliferative assay and was previously described in detail (30).

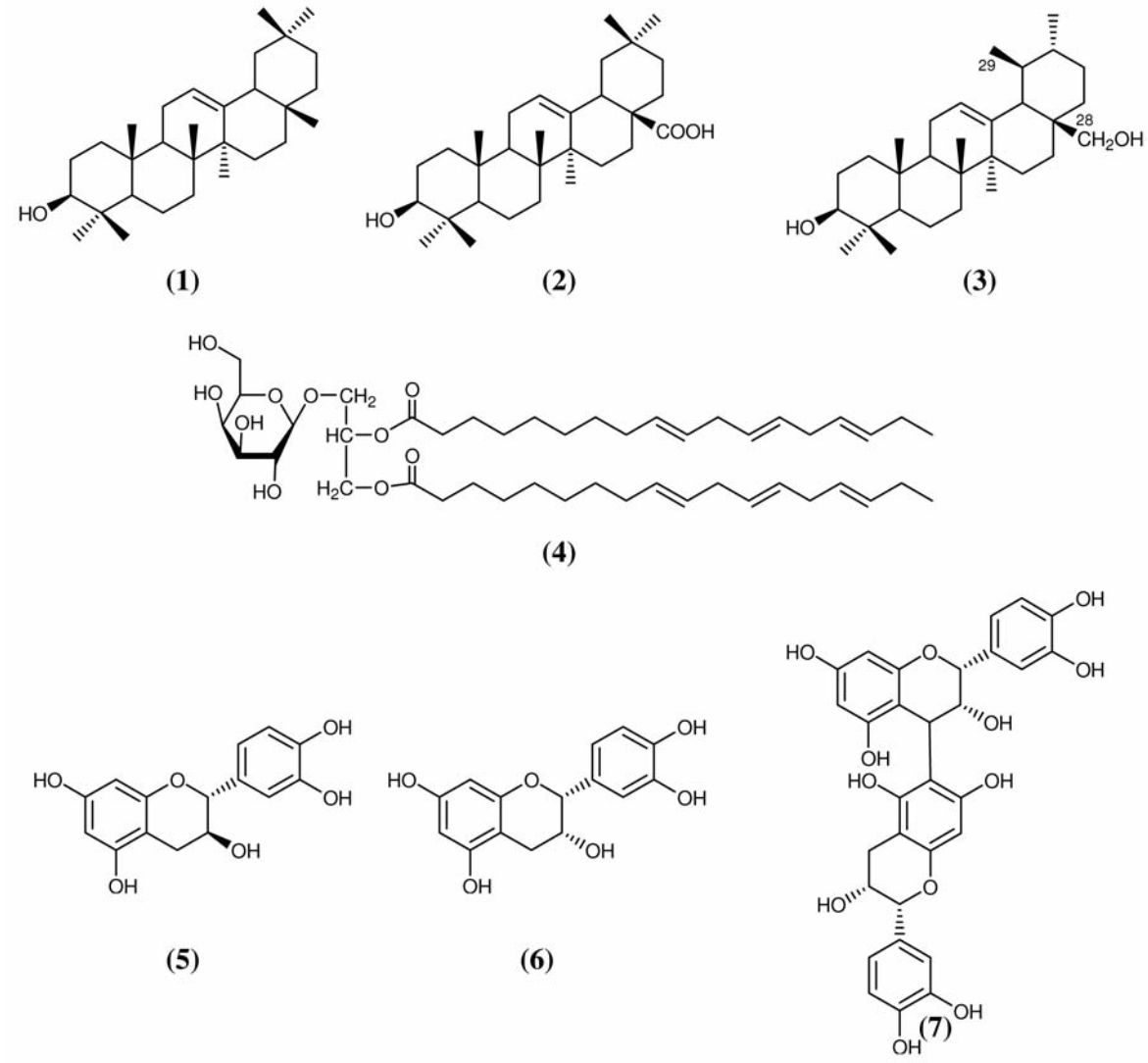

Figure 1. *Structures of the compounds isolated from the methanolic extract of C. edulis: β-amyrin (**1**), oleanolic acid (**2**), uvaol (**3**), monogalactosyldiacylglycerol (MGDG) (**4**), catechin (**5**), epicatechin (**6**) and procyanidin B5 (**7**).*

Flow cytometry. The activity of the fractions and pure compounds on the P-gp transporter of parental and *MDR1* transfected progeny was evaluated by following the accumulation of the fluorochrome rhodamine 123 as previously described in detail (31). Compounds that inhibit the P-gp transporter cause accumulation of rhodamine 123 that would otherwise be extruded in the absence of the active compound (4). Briefly, the cells were re-suspended to a final density of 1×10^6 cells in 0.5 ml of serum-free McCoy's 5A medium. Each aliquot of cells was individually treated with the purified compounds dissolved in DMSO (Sigma, Germany) and incubated for 10 min, after which rhodamine 123 (Sigma) was added to each tube to yield a final concentration of 5.2 μM; the samples were then incubated for 20 min at 37˚C, centrifuged at 2000 rpm for 2 min and the pellet re-suspended in 0.5 mL of PBS. The PBS washing procedure was repeated twice. The fluorescence of the samples was measured by flow cytometry (Becton Dickinson FACScan, BD, USA). Verapamil (Sanofi-Synthelabo) at 10 mg/L was used as the positive control (4).

Semi-automated fluorimetry. The modulation of P-gp activity of each compound was also assessed by the semi-automated ethidium bromide (EB) fluorometric method using a Rotor-Gene 3000™ thermocycler with real-time analysis software (Corbett Research, Sidney, Australia) as described elsewhere (32).

Results

The MeOH extract of the fresh leaves of *C. edulis* was extracted with *n*-hexane, chloroform and EtOAc. The organic phases were subjected to multiple chromatographic purification, including VLC, RPC, preparative TLC, HPLC and gel-filtration, to afford seven pure compounds (**1-7**). On the basis of atmospheric pressure chemical ionization–mass spectrometry (APCI-MS), electron spray ionization mass spectrometry (ESIMS), ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY,

Table I. *Antiproliferative activity as shown by the 50% inhibitory concentration (IC_{50}) of the compounds isolated from C. edulis.*

Compound	IC_{50} (mg/L)	
	PAR	MDR
β-Amyrin (**1**)	11	10
Oleanolic acid (**2**)	10	21
Uvaol (**3**)	7	13
Monogalactosyldiacylglycerol (**4**)	9	5
Catechin (**5**)	10	12
Epicatechin (**6**)	8	6
Procyanidin B5 (**7**)	nd	13

nd, Not determined, PAR parental L5178 cells, MDR *MDR1*-transfectes L5178 cells.

HSQC, HMBC and NOESY investigations, and by comparison of the spectral data with those published in the literature, the compounds were identified as β-amyrin (**1**) (33), oleanolic acid (**2**) (34), uvaol (**3**) (35, 36), monogalactosyldiacylglycerol (acyls=linolenoyl group; MGDG) (**4**) (37), catechin (**5**) (38, 39), epicatechin (**6**) (38, 39) and procyanidin B5 (**7**) (Figure 1) (40). To our knowledge, this is the first report on the isolation of compounds **1-7** from *C. edulis*.

The antiproliferative activity of the purified compounds on mouse lymphoma parental cells and human *MDR1*-transfected mouse lymphoma cells is summarized in Table I. All of the compounds reduced the proliferation of both parental and *MDR1*-transfected cells. However, whereas oleanolic acid and uvaol were more effective against the parental cells, MGDG and epicatechin were more effective against the MDR1-transfected cells. It should be noted that in this study, the parental cells are cancer cells and therefore, compounds that reduce the replication of these cells also merit attention.

The effect of the isolated compounds on the inhibition of P-gp and consequent accumulation of rhodamine 123 inside the cells was measured in terms of fluorescence (Fl) by flow cytometry in presence and absence of the compounds. The activity is presented as a fluorescence activity ratio (FAR), which is equal to: $FAR=(Fl_{MDR\ treated} - Fl_{MDR\ control})/(Fl_{PAR\ treated} - Fl_{PAR\ control})$. FAR values are summarized in Table II. The concentration of DMSO used in the assay was 4% and corresponds to the maximum percentage of DMSO used to dissolve the compounds and thus its final concentration in the assay. The P-gp modulator verapamil was used at a concentration of 10 mg/L as the positive control. The compounds isolated in this study were devoid of any cytotoxicity at the concentrations employed (data not shown). As evident from the FAR values of Table II in comparison to the verapamil positive control, uvaol demonstrated significant inhibition of P-gp.

Table II. *Fluorescence activity ratio (FAR) values for the isolated compounds at the two concentrations tested as well as of the DMSO control.*

Compound	Concentration (mg/L)	FAR
Verapamil	10	6.97
β-Amyrin (**1**)	4	nd
	40	1.26
Oleanolic acid (**2**)	4	1.26
	40	5.29
Uvaol (**3**)	4	40.93
	40	76.07
Monogalactosyldiacylglycerol (**4**)	4	1.16
	40	2.05
Catechin (**5**)	4	2.50
	40	2.93
Epicatechin (**6**)	4	1.16
	40	0.97
Procyanidin B5 (**7**)	4	0.89
	40	0.58
DMSO	(4%)	0.69

Table III. *Relative fluorescence factor (RFF) values for the isolated compounds at the two concentrations tested.*

Compound	Concentration (mg/L)	RFF
Verapamil	40	100.0
β-Amyrin (**1**)	4	4.9
	40	8.9
Oleanolic acid (**2**)	4	15.8
	40	8.8
Uvaol (**3**)	4	11.1
	40	18.4
Monogalactosyldiacylglycerol (**4**)	4	12.4
	40	6.9
Catechin (**5**)	4	4.9
	40	4.6
Epicatechin (**6**)	4	8.6
	40	9.9
Procyanidin B5 (**7**)	4	nd
	40	nd
DMSO	–(4%)	<1

nd, Not determined.

The activity of the compounds isolated from *C. edulis* was also evaluated with the aid of the automated EB method. In this method, a compound that inhibits an efflux system extruding the fluorescent substrate EB produces an increase of fluorescence as a consequence of the build up of EB within the cell. The use of two distinct methods that evaluate efflux reinforces the significance of the inhibitory activity noted for a given compound.

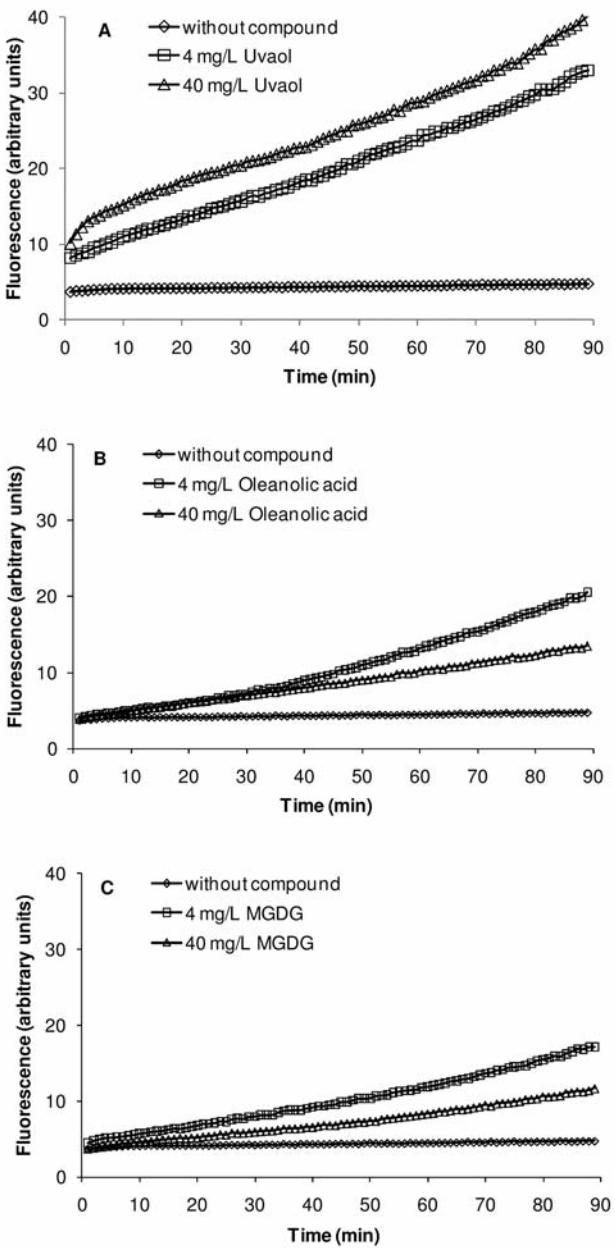

Figure 2. *Accumulation of ethidium bromide in MDR mouse lymphoma cells in the presence of A: uvaol, B: oleanolic acid and C: MGDG.*

The effects of oleanolic acid, uvaol and MGDG on the accumulation of EB by the mouse lymphoma transfected with *MDR1* are illustrated by Figure 2 which shows that these compounds promote the accumulation of the fluorochrome EB. These results confirm the effects of the compounds as evaluated by a totally different method, namely flow cytometry: uvaol, the most active, followed by oleanolic acid and MGDG.

The amount of fluorescence at the end of 90 minutes in the absence and presence of each compound is provided by Table III. Briefly, uvaol, the most effective compound that reversed resistance of the *MDR1*-transfected mouse lymphoma cells to the cytotoxic agent doxorubicin, produced the greatest degree of fluorescence, thereby indicating that this compound strongly inhibits the efflux activity of P-pg.

Because the combination of uvaol was the most active agent inhibiting the efflux activity of P-gp, the checker-board method was employed for the determination of any synergism between uvaol and doxorubicin on the replication of the *MDR1*-transfected mouse lymphoma cells. The checker-board method provides a FIX value that is obtained as the sum of the FIX values for each compound separately (41): $FIX=FIX_{uvaol}+FIX_{doxorubicin}$. The FIX value of each compound is the fractional IC_{50} of the combined drugs divided by their individual IC_{50} as follow: $FIX_{uvaol}=IC_{50(uvaol+doxorubicin)}/IC_{50uvaol}$ and $FIX_{doxorubicin}=IC_{50(uvaol+doxorubicin)}/IC_{50doxorubicin}$. If the FIX value is equal to 1, it represents an additive effect of the two drugs that is equal to the sum of the individual effect of each of the drugs when used separately. If the FIX value is equal to any of the individual values it means that the result with the two drugs does not significantly differ from that of the most effective drug used alone. FIX values larger than 1 represent antagonism and mean that the result of the two drugs is significantly less than the additive response. Synergism corresponds to FIX values equal to or less than 0.5 (41). Uvaol in combination with doxorubicin yielded a FIX value of 0.49, suggestive of synergism.

Discussion

The results obtained in this study show that all of the compounds isolated from *C. edulis* have an antiproliferative effect on both cell lines. However, the antiproliferative activity of oleanolic acid, uvaol and catechin is more pronounced on the parental cell line. The antiproliferative effect of β-amyrin and epicatechin is not significantly different for the two cell lines. However, the MDR cell line is a little more sensitive to MGDG. Previous workers showed that this compound reduced the biosynthesis of cholesterol by inhibiting the human enzyme lanosterol synthase (37). This result, together with the lipophilic nature of MGDG, suggests that MGDG acts in the MDR cell line in a more specific manner.

Of the compounds isolated from *C. edulis*, uvaol was the most effective in inhibiting the extrusion of rhodamine 123 and EB by the MDR mouse lymphoma cells as evident from the accumulation of these P-gp substrates, demonstrated by two distinctly different methods, flow cytometry and the EB automated method, respectively. Because this compound

does not produce toxicity at the concentrations employed, it may have potential as an adjuvant for the therapy of cancer that is refractory to therapy.

The other compounds isolated from *C. edulis* also inhibited the P-gp transporter of MDR cancer cells, but to a lesser extent than uvaol. This is the first time that the inhibitory activity of uvaol, β-amyrin, oleanolic acid and MGDG against the P-gp transporter of MDR cancer cells has been shown. Catechin, epicatechin and its derivatives, mainly found in green tea, have been isolated from other plants and were shown to act as chemopreventive (42) and anti-inflammatory agents (43), and inhibitors of gastric H^+, K^+-ATPase (44). We believe that these compounds are responsible for the traditional use of *C. edulis* for stomach problems and against different kinds of inflammations (22, 26, 27). Moreover, because these compounds have now been shown to inhibit the P-pg transporter, they may also have the capacity to inhibit other efflux pumps, such as those that are responsible for the excessive extrusion of H^+ by gastric epithelial cells. Analogues of epicatechin have been shown to inhibit P-gp, but the parental compound itself has been reported to be devoid of such activity (45). This variance between our results and those of others (45) may be due to the expression of the human P-gp transporter or its location on the plasma membrane of the mouse lymphoma cell which renders it immune to the action of catechin or epicatechin. Procyanidin B5 dimmers were also demonstrated as potential chemopreventive agents against breast cancer by suppressing *in situ* estrogen biosynthesis (46). MDGD, catechin and its isomer epicatechin are not responsible for the reversal of MDR activity exhibited by the plant extract, since they show low FAR values.

The structural difference between uvaol and β-amyrin or oleanolic acid is the methyl group at position C-29, which seems to play an important role in the activity of this class of compounds. Further studies should be conducted in order to detail the structure activity relationship. Furthermore, members of the triterpenes group may serve as lead compounds for the synthesis of new compounds that may prove even more effective as inhibitors of P-gp than those isolated from *C. edulis*, hence, they may serve as adjuvants in cancer chemotherapy.

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