Polyphenols Purified from the Brazilian Aroeira Plant (*Schinus terebinthifolius*, Raddi) Induce Apoptotic and Autophagic Cell Death of DU145 Cells

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Abstract. *Polyphenols extracted from many plants have shown antiproliferative and antitumor activities in a wide range of carcinogenesis models. The antiproliferative effects of polyphenols purified from the Brazilian aroeira plant (Schinus terebinthifolius, Raddi) were investigated on the androgen-insensitive DU145 human prostatic carcinoma cell line. A F3 fraction purified from leaf extract inhibited the DU145 cell proliferation more than 30-fold compared to the crude extract. By flow cytometric analysis, the polyphenol fraction was demonstrated to induce G_0/G_1 cell growth arrest and cell apoptosis. This apoptosis was evidenced by caspase 3 stimulation in F3-treated cells as compared to crude extract treated cells. The acid phosphatase activity of lysosomes was strongly activated in the lysosomal fraction of the F3-treated DU145 cells. This lysosomal activation, together with the appearance of autophagic vacuoles, suggests that "type 2 physiological cell death" was also involved in this antiproliferative effect. HPLC analysis of this F3 fraction showed 18 different subfractions. Among these subfractions, F3-3, F3-7 and F3-13 strongly inhibited DU145 cell proliferation in a dose-dependent manner. However, the nature of these polyphenols remains unknown since only one (Isoquercitrin) of the tested pure polyphenols co-migrated with F3-13. Since lysosomotropic drugs are considered as possible regulators of lysosome activity, aroeira polyphenols could target lysosomes of prostatic cancer cells to induce autophagic cell death.*

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Aroeira is a shrubby tree with narrow, spiky leaves. It grows 4 to 10 m tall and is still employed in herbal medicine today in Brazil as a bark tea and bark-and-leaf tea. Many of the plant's documented biological activities are attributed to the phytochemical compounds found in the plant.

Polyphenols constitute a group of approximately 4000 naturally occurring compounds ubiquitous in all vascular plants (19). They are found in several medicinal plants and herbal drugs used in traditional medicine around the world. Many studies have been conducted on the antiproliferative and antitumor activities of polyphenols (3, 8, 11, 20, 35, 40). Flavanoïds from green tea have been shown to possess cancer chemopreventive effects in a wide range of target organs in rodent carcinogenesis models (7, 12, 13, 15, 24, 25, 33, 36, 41). In addition, epidemiological studies indicate that people consuming green tea regularly may have a lower risk of prostate cancer (21, 26, 28, 34). The incidence of breast and prostate cancers is also low in Asian countries where a low fat diet and flavanoïd consumption are predominant (10, 14, 23, 29).

Green tea polyphenols present remarkable preventive effects against prostate cancer in mice and many of these effects are mediated by the ability of polyphenols to induce apoptosis of cancer cells (18, 22, 31, 39). Green tea polyphenols cause a G_2/G_1 phase cell cycle arrest and

apoptosis of both androgen-sensitive LNCaP and androgen-insensitive DU145 human prostate carcinoma cells (1, 2, 16, 18). A previous study suggested that the antiproliferative effects of quercetin and kaempferol can significantly contribute to alterations in the cell cycle progression of PC3 cells (27).

In the present study the effects of polyphenols purified from the Brazilian aroeira plant (*Schinus terebinthifolius*) on a prostatic cancer cell line were studied and the inhibitory effects of various purified fractions and pure flavanoïds on the androgen-insensitive DU145 prostatic cell line were compared.

Materials and Methods

Polyphenol preparation from the leaves of S. terebinthifolius. Aroeira powder (0.4 g) was boiled in 6 mL of water for 15 min. The mixture was then filtrated and centrifuged at 12000 g to give the crude extract. This crude extract was shacken with 2 volumes of chloroform for 5 min and let to decant in order to obtain the aqueous fraction 2.

Half of fraction 2 was extracted with 2 volumes of ethylacetate. After stirring, washing and drying at 65˚C, the aqueous fraction and the ethylacetate fraction were termed fraction 2'(F2') and fraction 3 (F3), respectively.

These fractions prepared, as summarized in Figure 1, were used for analytical and experimental biological experiments. The dry extracts obtained from 1 mL of each fraction were weighed. The different preparations gave an average concentration of 658.2±49.2 μg/mL in the F3 fraction obtained from the crude extract (0.4 g / 6 mL) (Table I).

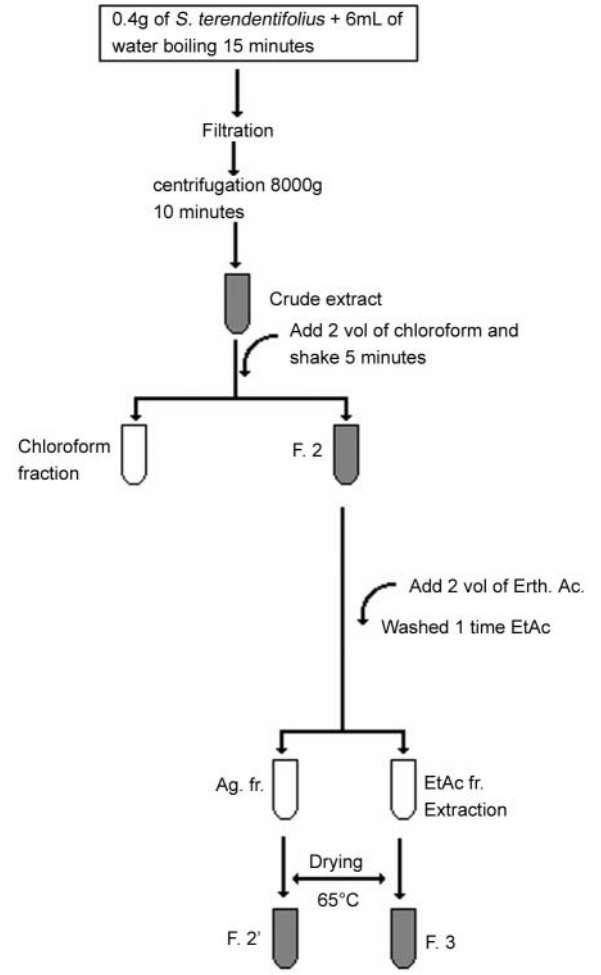

Figure 1. *Polyphenol purification.*

Cell culture and MTT test. The human prostatic DU145 cells were continually cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin and 10% foetal calf serum (FCS). The cells were cultured at 37˚C, 5% CO_2 in 100% humidity.

For experiments, the DU145 cells were cultured in 96-well microplates (Microtest™ Tissue Culture Plates, Falcon) at a density of $7.5x10^3$ cells per well in 100 μL of medium. After 24 h of culture, new medium was added with or without various concentrations of polyphenols. The cell number was quantified by MTT test after 48 h of culture. After harvesting the culture medium, 100 μL of the MTT solution (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) at 1 mg/mL were added for 10 min at 37˚C. The absorbance of the solution was measured in each well at 550 nm with a microplate DINATECH-Reader.

Lysosome preparation and acid phosphatase activity. After trypsinisation and centrifugation, $1.8x10^6$ subconfluent cells were resuspended in 2 mL of homogenisation buffer (TRIS 10 mM, EDTA 0.2 mM, KCl 10 mM, saccharose 250 mM, pH 7.4) at 4˚C. The cells were homogenised with Potter-Elvejhem at 250 rpm for 1 min in an ice bath.

The lysosome fractions were isolated by fractionated centrifugation at 4˚C in a Beckman centrifuge (J2-21 type) equipped with a JA-20 rotor, according to Rodrigues *et al.*, 1998. Homogenates were centrifuged for 10 min at 900xg to eliminate intact cells, membranes and nuclei. The supernatants were centrifuged for 10 min at 1500 g and then for 20 min at 12000 g. New supernatants were finally centrifuged for 20 min at 32000 g. This supernatant was used to measure acid phosphatase and protein contents. The pellet containing lysosomes was resuspended in 500 μL of homogenisation buffer. Half of this lysosome preparation was treated with Triton X-100 at a final concentration of 0.1% (v/v) for 20 min over ice and centrifuged again at 32.000xg to separate intact lysosomes. The acid phosphatase activities were measured as previously described (32).

SDS-PAGE and Western blot. The cell pellets were resuspended in a RIPA lysis buffer of 50 mM Tris-HCl, pH 7.4, [150 mM NaCl, 10% NP-40, 2.5% w/v deoxycholic acid, 0.95 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, PMSF; 50 mg/mL aprotinin (Sigma Chemical; St. Louis, MO, USA), 1 mM pepstatin at 1 μg/mL 50 mM leupeptin (Bayer; Leverkusen, Germany)]. The contents were incubated at 4˚C for 20 min. DNA was sheared by passing the lysate through a 26 gauge needle. The cell supernatants were obtained following centrifugation at 3000xg for 20 min at 4˚C, and were

Table I. *IC_{50} values of the crude extract and HPLC-purified fractions.*

Fractions	CE (554 μg)		F2 (24.4 μg)		F3 (13.2 μg)	
	IC_{50} (μg/mL)	%	IC_{50} (μg/mL)	%	IC_{50} (μg/mL)	%
Peaks	>200	100	200	100	8	100
3	>200	12.5	10	10.1	3.1	17.5
7	>200	3.1	3	2	0.64	3.2
13	>200	7.1	3	6.3	2.7	16.5

Twenty μL of crude extract (A), F2' (B) or F3 (C), were injected into a phase reverse HPLC column equilibrated in 95% acetic acid (0.2% in H_2O) and 5% methanol and were eluted by a discontinuous gradient of methanol as indicated in the Materials and Methods section. For each run, 18 fractions were recovered and tested for their ability to inhibit tumour cell proliferation.

collected and stored at –80˚C until use for protein assay and Western blot analysis. The protein concentration in the total cell lysates was quantified by the Bradford method (4).

The samples were incubated at 60˚C for 10 min in a loading Laemmli buffer before running (30). Each lane was loaded with 20 μg of total proteins which were separated by 13% SDS-PAGE under reducing conditions. After electrophoresis, fractionated proteins were transferred onto a 0.45 μm pore PVDF membrane (Millipore Immobilon-P) at 100 V for 2 h in a Bio-Rad mini Trans-Blot electrophoretic transfer cell. The blotted membrane was rinsed with PBS and then blocked with PBS pH 7.4 (w/o calcium, w/o magnesium, Gibco) containing 5% (w/v) non-fat milk and 0.1% (v/v) Tween-20 at 4˚C, overnight.

The membrane was then incubated with mouse anticaspase 3 and anti α-tubulin monoclonal antibody (Polyclonal rabbit, CPP32, PharMingen) diluted to 1:5000 (v/v) in PBS containing 5% (w/v) non-fat milk. The membranes were then incubated with horseradish peroxidase conjugate rabbit-anti-mouse IgG (Jackson Immuno Research Laboratories, Inc.) diluted to 1:10000 (v/v) for 1 h at room temperature. After the final wash, the antibody-antigen complexes on membrane were revealed by ECL Western blotting detection reagents (Amersham).

HPLC analysis. The HPLC analyses were conducted on a Beckman Gold system chromatograph fitted with a C-18 reversed phase column (25 cm x 4.6 mm). For the separations of individual compounds in the crude extract and fractions F2 and F3, the mobile phase consisted of 2% acetic acid in doubly distilled water (solvent A) and methanol (solvent B), utilising the following gradient over a total run time of 45 min: 95% A for 2 min, 75% A in 8 min, 60% A in 10 min, 50% A in 10 min and 0% A, until completion of the run (Montedoro and Petrucioli *et al.* 1992; and Servili *et al.*, 1999). The extracts (20 μL) were injected into the HPLC. The phenolic compounds in the eluants were detected with a UV diode-array detector 168 (Beckman) set at 280 and 360 nm. The flow rate of the mobile phase was 1 ml/min. Standard purified phenol compounds (quercitrin, caffeic acid, citric acid, isoquercitrin, resveratrol, rutine, eriodictyol, quercetin, kaemferol, quinic acid, apigenin) were dissolved to 4 mM in methanol and injected into the HPLC.

To isolate the active compound in F3, 10 HPLC runs were performed and 18 major peaks were collected. The solvent was removed by lyophilisation. The different peaks were solubilised in 200 μL of PBS and tested for their biological activity.

Flow cytometer analysis. The human prostatic DU145 cells were cultured at a density of $5x10^5$ cells per 25 cm^2 culture vessels (T-25). After 24 h of culture, new medium was added with the F3 extract. The cells were rinsed in PBS, treated with trypsin and numbered. The pellet was rinsed in PBS and fixed in 70% ethanol for at least 2 h on ice.

The cells were centrifuged for 5 min at 200 g, then pelleted, rinsed in 5 mL PBS and recentrifuged. The cell pellet was suspended in 1 mL of propidium iodide (5 μg PI, 250 μg ribonuclease A, 500 μg tri-sodium citrate, 0.15% Triton X-100) staining solution and was incubated for 15 min at 37˚C.The reaction was blocked by adding 0.1 mL of sodium chloride at 9.9% (w/v).

Cell sorting was performed using a FACStar flow cytometer (Becton Dickinson) as previously described (9). Cell distributions with different cell DNA contents were analysed with Cell Quest software. The red (FL2) fluorescence emission, corresponding to the propidium iodide (PI) staining, was recorded on a 4-decade logarithmic scale. A minimum of 10,000 events was analysed from each sample.

Results

Inhibitory effects of the S. terebinthifolius crude extract and purified fractions on DU145 cell proliferation and viability. The effects of the crude extract and purified fractions F2, F2' and F3 on the DU145 cell proliferation and viability were measured using the MTT test. Experiments with increasing amounts of each fraction showed that crude extract, F2 and F3 inhibited the proliferation of DU145 cells dose-dependently, whereas F2' had no effect (Figure 2A, B). This inhibitory effect reached a maximum after 48 h of treatment.

By calculating the IC_{50} for each fraction, the F3 with a IC_{50} of 8 μg/mL was found to be the most potent inhibitory fraction since the IC_{50} measured from the crude extract and F2 were above 200 μg/mL (Table I).

Polyphenol F3-induced D145 cell apoptosis. Analysis of the DNA content by flow cytometric analysis showed the DU145 cell distribution in the G0/S/G2M phases (Figure 3A). Treatments of the cells with the F3 fraction (0.20 μg/mL)

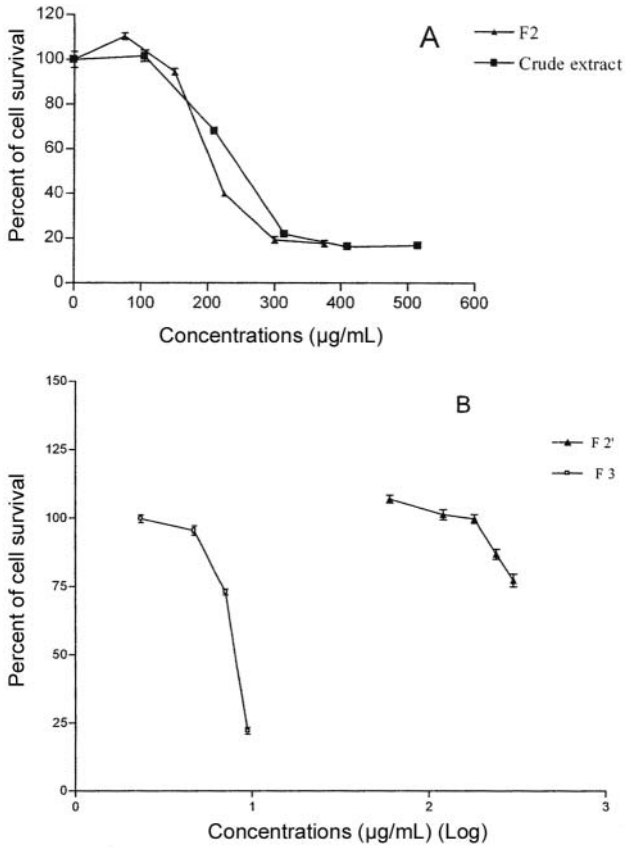

Figure 2. *Effect of increasing concentrations of crude extract and various fractions on the DU145 cell proliferation. A: DU145 cells were treated for 48 h with different concentrations of the polyphenol crude extract and the percentages of cell survival were determined by the MTT test as described in the Materials and Methods. The DU145 cells were treated for 48 h with increasing concentrations of purified F2, obtained as described in Figure 1. B: Increasing concentrations of the F2' and F3 fractions purified from the crude extract (Figure 1) were added to the DU145 cells for 48 h and the percentage of cell survival was measured as previously described. The data were expressed as the percentage (as compared to the controls) of the MFI±SEM of 3 separate experiments.*

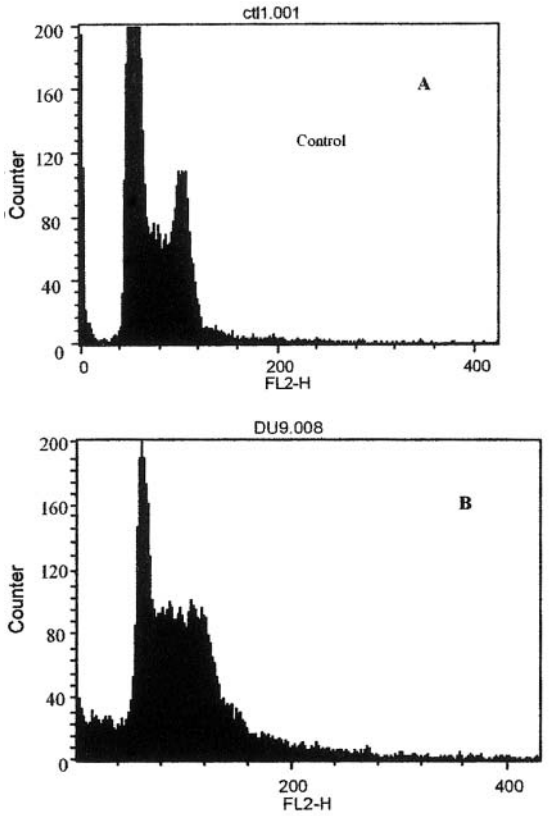

Figure 3. *Flow cytometric analysis of DNA content from DU145 cells treated with the F3 fraction. Flow cytometric analysis of (A) untreated cells and (B) cells treated for 48 h with 0.20 µg/mL of the F3 fraction.*

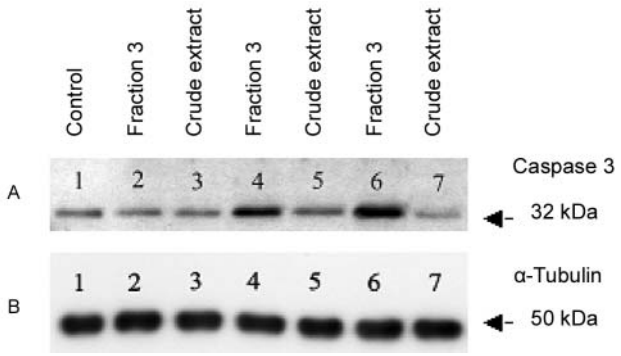

Figure 4. *Caspase 3 synthesis after Fraction 3 and crude extract treatments of DU145 cells. Caspase 3 was identified by immunobloting as described in the Materials and Methods section. A: control untreated cell (1); cells treated with Fraction 3 for 4 h (2), 24 h (4) and 48 h (6); cells treated with crude extract for 4 h (3), 24 h (5) and 48 h (7). B: immunobloting was probed as described with the α-tubulin antibody as the standard.*

induced cell apoptosis and cell aggregation (Figure 3B), as evidenced by the analysis of caspase 3 synthesis in the treated cells compared to the untreated control (Figure 4). Fraction 3 strongly induced the caspase 3 synthesis, indicating an induction of apoptosis whereas the crude extract did not produce an effect.

Effect of F3 on the lysosome activation in DU145 cells. Since lysosomes are potential targets for polyphenols (42) lysosome activation, measured by its phosphatase acid activity, was measured.

We observed cell proliferation arrest and the appearance of dead cells after 48 h of treatment. The percentage of dead cells increased by 22.6% after treatment with F3 (Table II). This pro-apoptotic effect of F3, not observed with other fractions, was also evidenced by a decrease of soluble proteins in the cytosolic fraction.

Table II. *Effect of F3 from S. terenbintifolius extract on the lysosome activation in DU 145 prostatic tumor cells.*

Cells - Proteins and Lysosome Activities		Control[a]	Treatment with Fraction 3[a]
Dead cells (%)[b]	Dead/(alive + dead)	7.68	22.61
Soluble proteins (μg/mL)[c]	Cytosolic fraction	452.28±56.29	364.13±30.85
	Lysosome surpernatent	52.03±4.32	54.56±10.28
Acid phosphatase specific activities (mU/mg of proteins)[d]	Cytosolic fraction	45.25±1.69	38.24±0.34
	Lysosome fraction per protein of homogenate	29.60±0.83 (0.65)[e]	42.35±0.96 (1.11)
	Lysosome fraction per protein from Triton-treated lysosomes	42.44±2.46 (0.94)	66.67±2.06 (1.74)

[a]Each value of control and treatment represents mean±sd of 9 independent measures. $p<0.05$
[b]Dead cells were measured by the trypan blue exclusion test as described in the Materials and Methods section.
[c]The soluble protein amount was measured by the Bradford test.
[d]Acid phosphatase activities were determined as described in the Materials and Methods section.
[e]Numbers in brackets are the ratio of acid phosphatase activities in lysosome fractions to total cytosolic fraction from untreated (control) and Fraction 3-treated DU 145 cells.

In contrast, the phosphatase acid specific activities in the lysosome fractions, both before and after Triton treatment, were significantly increased by F3 treatment (Table II). Thus, the polyphenols from F3 could induce a apoptosis in the DU145 cells, with the observed lysosomal phosphatase acid activation suggesting an autophagic process.

Analysis and purification of the crude extract, F2' and F3 from S. terebinthifolius. The polyphenols contained in the crude extract were separated by HPLC. The pattern of crude extract HPLC analysis (Figure 5A) was divided into 18 major peaks. The retention times of the obtained peaks were compared to those of the purified polyphenols. Under the same conditions, the F2' and F3 patterns showed similar peaks but with different absorbances (Figure 5B). However the HPLC analysis of F3 (Figure 5C) evidenced the absence of peaks 1 and 2 as well as the enrichment of some polyphenols as compared to others. Thus, the relative amount of peak 13 is higher in F3 as compared to F2'. Simultaneous runs of F3 with purified compounds showed peak 13 (RT 26.92 min) eluted between citric acid (RT: 25.92 min) and rutin (RT: 30.84 min) co-migrated with isoquercitrin (RT: 28.31±1.29) (Table III).

Table III. *Elution of pure phenolic compounds by HPLC.*

Phenolic compound	Retention time (min)
Quercitrin	2.96
Caffeic acid	19.36
Citric acid	25.92
Isoquercitrin	27.03
Resveratrol	28.72
Rutin	30.47
Eriodictyol	33.75
Quercetin	36.17
Kaepferol	39.99
Quiniq acid	40.27
Apigenin	40.51

Effect of HPLC-purified fractions on DU145 cell proliferation and viability. The polyphenols from the abore mentioned 18 peaks were concentrated and tested for their ability to inhibit DU145 cell proliferation, to demonstrate the differential effects of the subfractions. Thus, the subfractions found in both F2' and F3 can weakly or strongly inhibit DU145 cell proliferation (peaks 3, 7, 8, 11, 12, 14 and 15) (Figure 5D, E). But the subfraction 13, enriched in F3, was a more potent inhibitor than the other subfractions and this inhibition was dose-dependant (Figure 6). Subfractions 3 (IC_{50} of 3.1 μg/mL) and 7 (IC_{50} of 0.64 μg/mL) also showed a dose-dependant inhibition of DU145 cell proliferation (Figure 6). Other subfractions did not show an inhibitory effect dependant of the concentration.

In order to compare the HPLC purifications of these different polyphenols, the percent yield of each peak area calculated by HPLC software, was ascribed to the total surface of the A, B and C column elutions (Table III). By comparing the IC_{50} values of each subfractions, it can be seen that inhibitory effect of F3.3 was similar to that of the F3 fraction (3.1 μg/mL). In contrast, polyphenols from the F3-7 and F3-13 peaks, with 3-fold lower IC_{50}, inhibited DU145 cell proliferation to a greater extent.

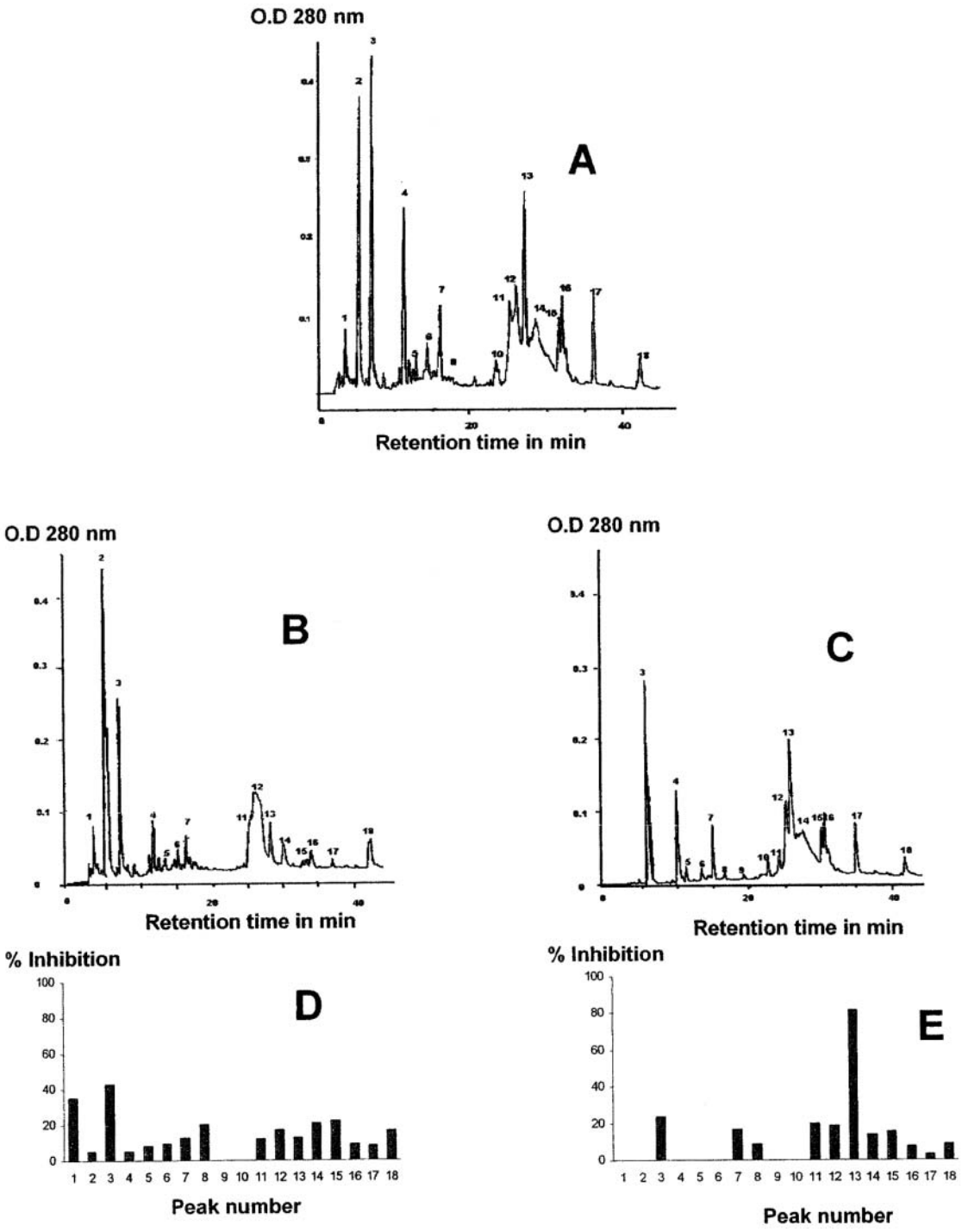

Figure 5. *HPLC purifications of the crude extract (A), F2' (B) and F3 (C). Subfraction inhibition of F2' (D) and F3 (E).*

Since F3-13 polyphenol was eluted close to pure isoquercetrin, the effect of increasing concentrations of this polyphenol on DU145 cell proliferation was tested. Isoquercitrin dose-dependently inhibits DU145 cell proliferation as measured by the MTT test, whereas citric acid had no significant effect on proliferation (Figure 7). The IC_{50} values of the isoquercitrin effect was similar to that of the F3-13 fraction.

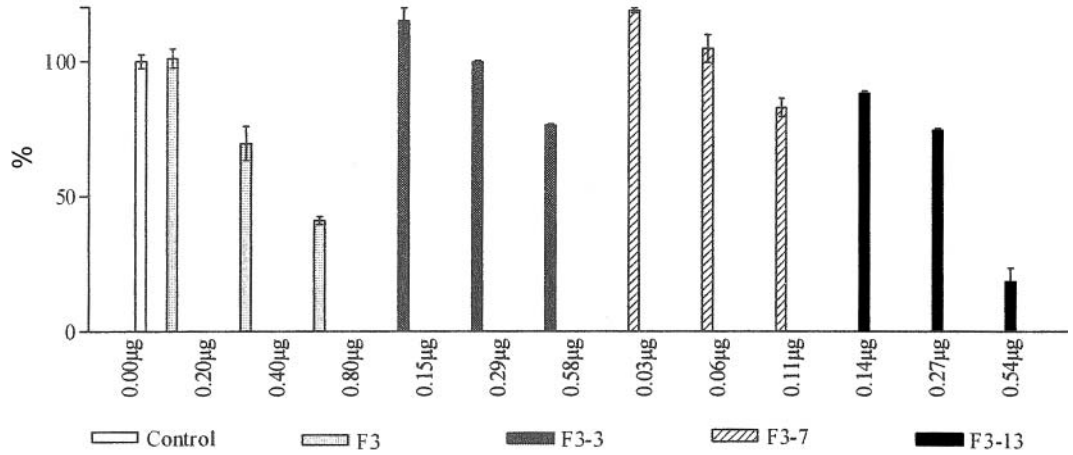

Figure 6. *Inhibitory effects of the F3 and F3 HPLC subfractions on DU145 cell proliferation. As compared to the control, the cell proliferations were measured with the MTT test in the presence of 3 increasing contrations of the F3 fraction, the F3-3, F3-7 and F3-13 subfractions were obtained and concentrated as described in the Materials and Methods. Each measure was performed in triplicate ±SEM. When 554 µg of CE were loaded on column A, 69.3 µg and 39.1 µg were recovered in peaks 3, 7 and 13, respectively. After applying 24.4 µg of F2 fraction to column B, 2.44 µg, 0.50 µg and 1.53 µg were recovered in peaks 3, 7 and 13, respectively. Similarly, 13.2 µg of F3 fraction gave 0.58 µg, 0.11 µg and 0.54 µg in the 3, 7 and 13 subfractions, respectively.*

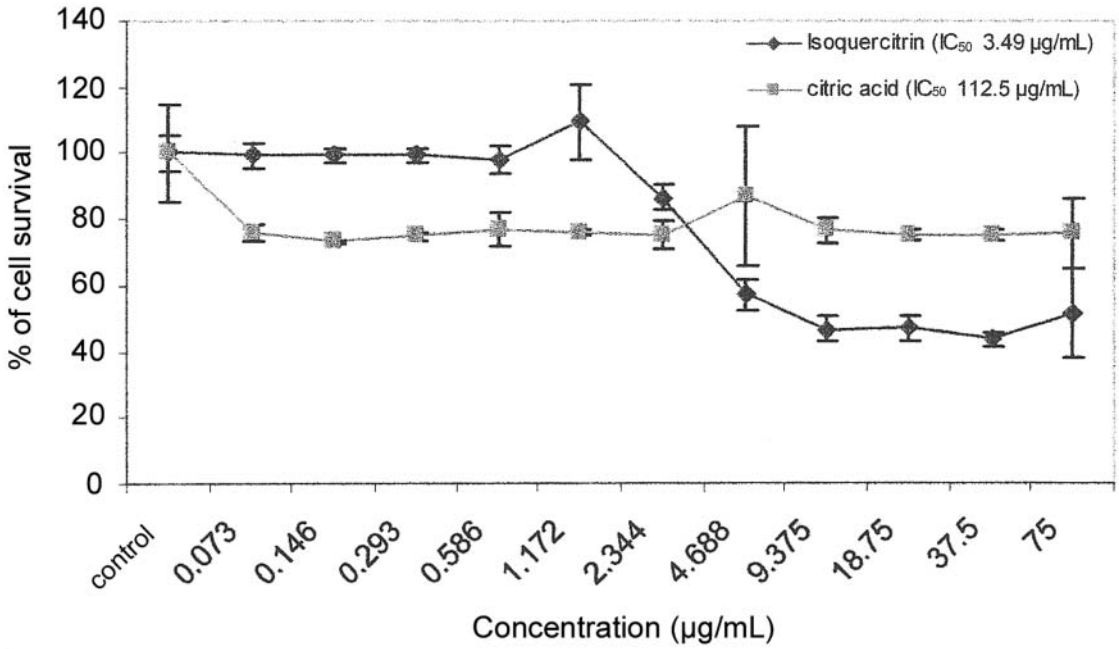

Figure 7. *Inhibitory effect of isoquercitrin and citric acid on DU145 cell proliferation. Cell proliferation was measured by the MTT test as described in the Materials and Methods section.*

Discussion

We investigated the effect of polyphenols purified from the Brazilian aroeira polyphenols on 3 human prostate cancer DU145 cell lines.

We showed that *S. terebinthifolius* polyphenols caused cell growth arrest and apoptosis in the androgen-insensitive DU145 prostate carcinoma cells. A similar antiproliferative effect was observed on the oestrogen-insensitive breast cancer MDA-MB 435 and the androgen-sensitive LNCaP prostate cell lines.

The number of DU145 cells having nuclei with chromatin condensation increased after 24 h of treatment. These cells showed shrinkage of both their cytoplasm and nuclei,

indicative of apoptotic programmed cell death. Dense bodies and autophagic vacuoles with limiting membranes present in these cells suggest that "type2 physiological cell death" or "autophagic degeneration" is also involved in these death effects. These results are in agreement with previous showing that in LNCaP and DU145 cells which are cell cycle-arrested, occurred cytoplasmic changes indicative of type 2 (autophagic) non-apoptotic programmed cell death (5, 37).

It has also been shown that autophagy was involved in PC12 cell death following serum deprivation and that this type of cell death is a lysosomal enzyme downstream autophagic process (38). Moreover, in autophagy cytoplasmic vacuoles are mainly derived from lysosomes (6). Here, we showed that *S. terebinthifolius* polyphenols stimulate acid phosphatase lysosomal activities in DU145 cells, suggesting the participation of the lysosomal compartment in the observed programmed cell death. However, apoptotic cell death and autophagic cell death are not mutually exclusive, but rather appear to reflect a high degree of flexibility in a cell's response to changes in environmental conditions.

Depending on the polyphenol concentrations, we observed either stimulation or inhibition of DU145 cell proliferation. These results can be interpreted as the polyphenol effects on several targets in DU145 cells. For example, one polyphenol at certain concentrations could stimulate a proliferative signalling pathway and at higher concentrations could inhibit this or another signalling pathway. Thus, a dynamic balance between positive and negative signals could explain these biphasic effects. In this context, the F3-13 and F3-7 polyphenols may be mainly responsible for the inhibition observed with the F3 fraction and may shift the balance toward the inhibitory signals.

We cannot exclude the possibility of additive and/or synergistic effects of several polyphenols. Thus, the associations of those polyphenols present in the F2' fraction (peak 1 and 2) could mutually modulate the inhibitory and stimulatory signals to give the resulting weak activity of the F2 fraction.

A final possibility is that minor modifications of polyphenols not seen by HPLC analysis could modulate the antiproliferative activity on DU145 cells. This hypothesis could explain the contradictory results on the biological activities of these compounds.

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