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Roots of *Daphne gnidium* L. inhibit cell proliferation and induce apoptosis in the human breast cancer cell line MCF-7

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Daphne gnidium L. is a well-known Moroccan plant with cancer-related ethnobotanical use. In order to systematically evaluate its potential activity in breast cancer, four extracts from this plant of different polarity were tested for their antiproliferative effects on MCF-7 cells. The second aspect of this study related to understanding the nature and mechanism of the antiproliferative effect. Results from a viability assay showed the potent antiproliferative capacity of the hexane (IC_{50}-48 h: 630 $\pm$ 16 μg/ml), dichloromethane (IC_{50}-48 h: 112 $\pm$ 7 μg/ml) and ethyl acetate extracts (IC_{50}-48 h: 263 $\pm$ 9 μg/ml). On the other hand the methanol extract was inactive. LDH test revealed the cytotoxicity of the hexane extract as opposed to two others. The characterization of the ethyl acetate extract showed its dose-dependent pro-apoptotic effect. Surprisingly, we observed that activation of the inducible cyclooxygenase-2 followed the kinetics of apoptosis development. On the other hand, the dichloromethane extract showed a distinct effect on COX-2 activity as a function of the used dose. A low dose seemed to inhibit COX-2 activity whereas a high dose seemed to increase it. These findings suggest that *Daphne gnidium* L. might be of potential chemopreventive interest. Other studies are in hand to isolate the active agents responsible for the antiproliferative effect.

1. Introduction

Since 1961, several anti-cancer drugs have been put on the market that trace their origins to plants, such as taxol, oncovin, navelbine and vumon (Pezzuto 1997; Kinghorn et al. 1999; Lee 1999). Evidence accumulated to date has established that many chemotherapeutic agents effect kill tumour cells through launching the apoptosis mechanism (Fan et al. 1998; Hunnun et al. 1997; Lee et al. 2003). Apoptosis is a fundamental process for the normal development and homeostasis of tissues and organs in pluricellular organisms. Apoptosis was originally characterized by morphological features such as membrane blebbing, cell shrinkage, chromatin condensation, nucleosomal fragmentation and formation of apoptotic bodies. Cyclooxygenases (COX) are key enzymes in the conversion of arachidonic acid into prostanoids [prostaglandins (PGs), prostacycline and thromboxane] which are involved in apoptosis, inflammation, mitogenesis and immunomodulation. Two isoforms of COX, designated COX-1 and COX-2, have been identified: COX-1 is constitutively expressed in most tissues and seems to be responsible for housekeeping roles for normal physiological functions, while COX-2 is expressed at very low basal levels, but is induced by different substances such as pro-inflammatory cytokines, growth factors and tumor promoters, implying a role for COX-2 in both inflammation and control of cell growth (Hong et al. 2002). Thus, compounds that inhibit the activity or expression of COX-2 might be an important target for cancer chemoprevention or antiinflammation therapy.

Daphne gnidium L. belongs to the botanical family of Thymeleacees. It is very widespread around the Mediterranean basin. In Morocco, the roots and leaves of this plant have been traditionally used to avoid hair loss, as a purgative, odontalgic, as a hair tonic and to treat jaundice and several solid tumours (Hmamouchi 1999; Merzouki et al. 2000). A previous Algerian study on this plant showed marked antileukemic activity (Bouafia et al. 1994).

Our study was designed to evaluate the antiproliferative effect of four extracts from *Daphne gnidium* L. of different polarity on the MCF-7 breast cancer cell line. Another aim of the study was a better understanding of the mechanisms involved in the effects of *Daphne gnidium* L. in order to confirm or dismiss its cancer-related ethnobotanical use.

2. Investigations and results

2.1. Yields of extracts

The percentage yields of the organic extracts were hexane (0.72%), dichloromethane (3%), ethyl acetate (7.09%) and methanol (18.11%).

2.2. Daphne gnidium L. extracts and cell proliferation

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test showed that the hexane, dichloro-

methane and ethyl acetate extracts decreased MCF-7 cell proliferation at 24, 48 and 72 h compared to control (100%) (Fig. 1 A, B and C). On the other hand, the methanol extract did not show any significant activity (data not shown).

The concentrations of *Daphne gnidium* L. extracts required to produce a 50% reduction in cell proliferation (IC_{50}) were calculated by regression analysis using data from the MTT assays (Fig. 1 A, B and C). For the three bioactive extracts, the value of IC_{50} decreased with treatment time. The dichloromethane extract was the most active extract at 48 and 72 h of treatment (IC_{50}: 112 μg/ml for both treatment times, Fig. 1B), followed respectively by the ethyl acetate extract (IC_{50}: 263 μg/ml and 246 μg/ml at 48 and 72 h, Fig. 1C) and the hexane extract (IC_{50}: 630 μg/ml at 48 h and 500 μg/ml at 72 h, Fig. 1A). Thus, the compound responsible for the maximum antiproliferative effect seemed to be concentrated in the dichloromethane extract.

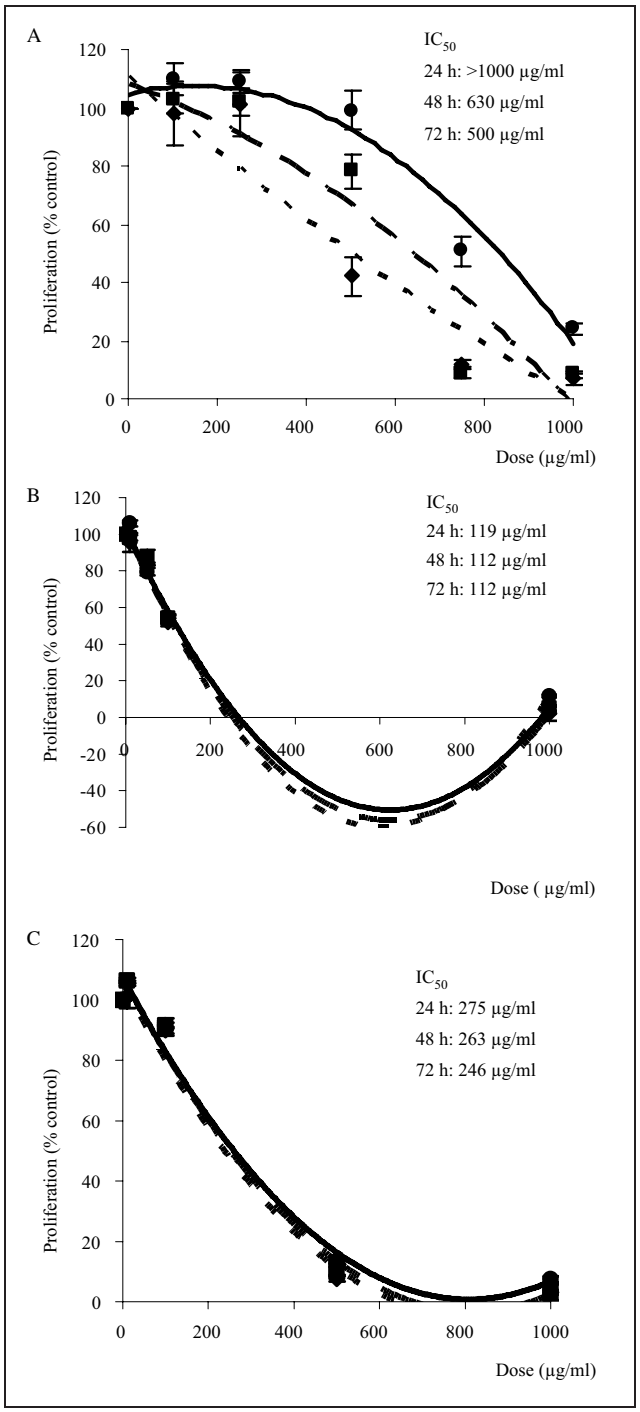

Fig. 1: Effect of *Daphne gnidium* L. extracts on MCF-7 cell proliferation. Cells were treated with various concentrations for 24 (—), 48 (**- -**) and 72 h (. . .). (A) MCF-7 cells treated with hexane extract. (B) MCF-7 cells treated with dichloromethane extract. (C) MCF-7 cells treated with ethyl acetate extract. Results presented as percentage of control (untreated cells) proliferation. Values expressed as mean ± SD of six experiments (p-value relative to control group: $p < 0.05$)

Analysis of morphological changes of MCF-7 cells following treatment with extracts at concentrations of 500 and 1000 μg/ml for 24 and 48 h, agrees with the results of the MTT test (Fig. 2 A, B, C, D and E; only photomicrographs of cells treated with doses of 1000 μg/ml for 48 h shown). MCF-7 cells treated with the hexane extract at concentrations of 500 and 1000 μg/ml showed lysis which was accentuated at 48 h (Fig. 2A). For the dichloromethane extract at 500 μg/ml and 1000 μg/ml, we mostly observed membrane blebbing, cell shrinkage and condensation of the nucleus (morphology related to apoptosis) (Fig. 2B). The ethyl acetate extract treatment induced slight condensation of the cytoplasm and nucleus at 24 h and clear condensation (advanced stage) at 48 h for the two concentrations tested (Fig. 2C). As regards the methanol extract of *Daphne gnidium* L., the morphological aspect of treated cells (Fig. 2D) was similar to control cells (Fig. 2E) for the two treatment times.

2.3. Cytotoxicity assay

In order to better characterize the effect of *Daphne gnidium* L. extracts on MCF-7 cell proliferation, plant extract-treated cells were subjected to a cytotoxicity assay [Lactate deshydrogenase (LDH) test]. The LDH test quantifies the LDH activity released from the cytosol of damaged cells into the culture supernatant to evaluate the percentage of lytic cell death compared to the maximum cell lysis obtained by Triton X-100 treatment. When MCF-7 cells were treated with the dichloromethane and ethyl acetate extracts at concentrations of 500 (average dose) and 1000 μg/ml (raised dose, almost completely lethal), no cytotoxic effect was detected. However, when 500 and 1000 μg/ml of the hexane extract were added, the percentage of cytotoxicity was raised 60% compared to control. Consequently, cell death induced by the hexane extract seemed to be necrosis (Table).

Table: Cytotoxic effect of *Daphne gnidium* L extracts on MCF-7 cells

	Tested concentrations	Cytotoxicity (%)	
		24 h	48 h
Control	–	0	2.57 ± 0.06
Hexane extract	500 μg/ml	60.33 ± 4.31	60.72 ± 4.42
	1000 μg/ml	71.01 ± 5.10	64.15 ± 4.49
Dichloromethane	500 μg/ml	0	0
extract	1000 μg/ml	0	0
Ethyl acetate	500 μg/ml	0	0
extract	1000 μg/ml	0	0

Cells treated with indicated concentrations of hexane extract, dichloromethane extract and ethyl acetate extract for 24 and 48 h. Results presented as percentage of positive control of kit. Each value represents mean ± SD of three separate experiments

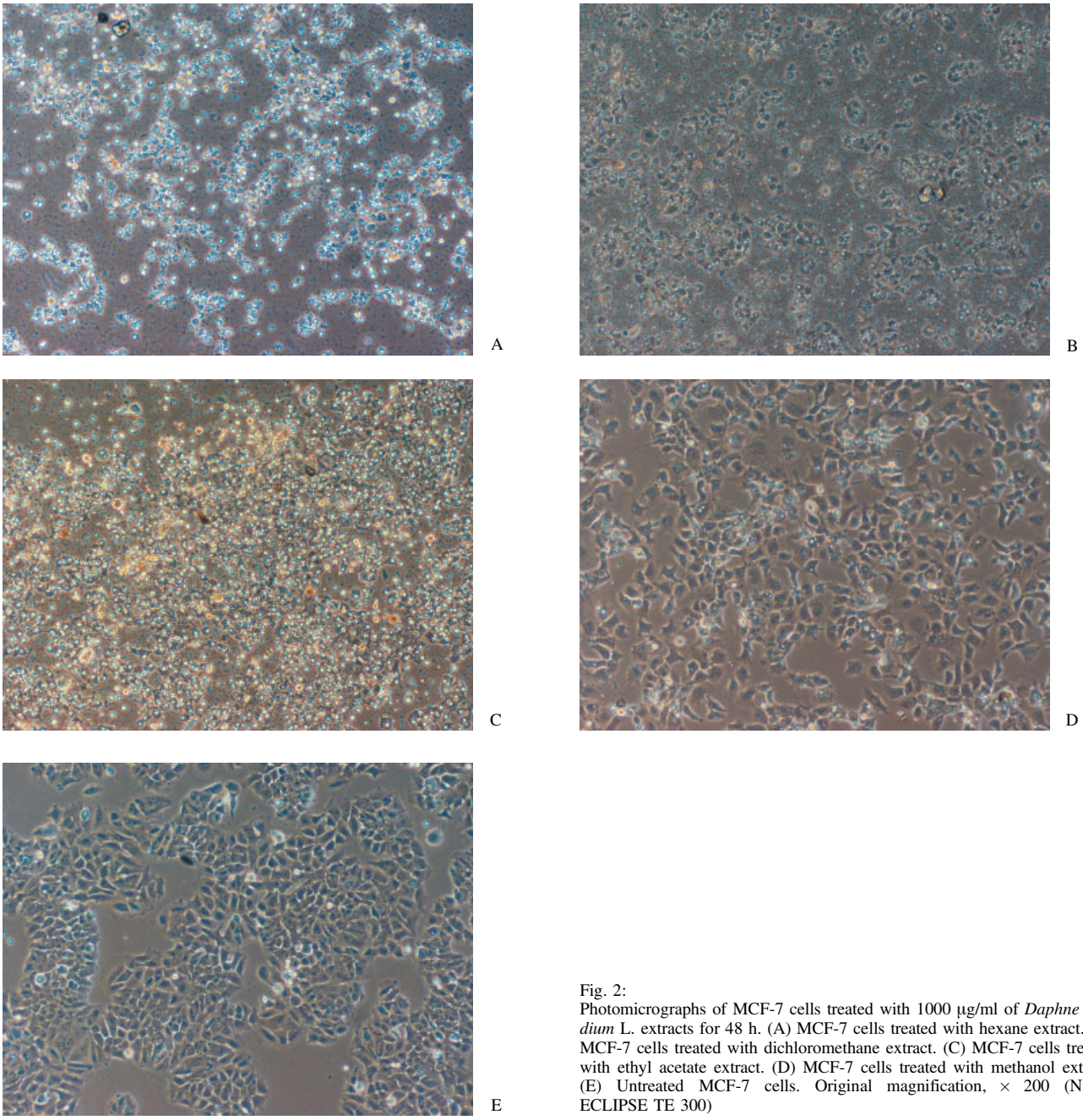

Fig. 2:
Photomicrographs of MCF-7 cells treated with 1000 μg/ml of *Daphne gnidium* L. extracts for 48 h. (A) MCF-7 cells treated with hexane extract. (B) MCF-7 cells treated with dichloromethane extract. (C) MCF-7 cells treated with ethyl acetate extract. (D) MCF-7 cells treated with methanol extract. (E) Untreated MCF-7 cells. Original magnification, × 200 (Nikon ECLIPSE TE 300)

2.4. Quantification of apoptosis

For this study, we retained the two non toxic extracts, that is the dichloromethane and ethyl acetate extracts, in order to determine whether the antiproliferative effect on breast cancer cells is related to programmed cell death induction. Apoptosis was evaluated by "cell death" enzyme-linked immunosorbent assay (ELISA) kit performed on a pooled cell fraction (floating and adherent cells). Treatment of MCF-7 cells for 24 and 48 h with the ethyl acetate extract at concentrations of 500 and 1000 μg/ml showed an increase in the apoptotic ratio of 1.7 and 2.6 times respectively compared to control (Fig. 3). However apoptosis was not detectable with the dichloromethane extract for the two times examined (Fig. 3). At this stage of our study, the results demonstrated that the ethyl acetate extract of *Daphne gnidium* L. induced apoptosis in MCF-7 in a dose dependent manner.

2.5. Assay of PGE_2 production

In addition, the two antiproliferative and non toxic extracts of *Daphne gnidium* L. were tested for their potential to inhibit COX-2 activity (inhibition of PGE_2 production) on cultured MCF-7 cells.

It appears from this test that PGE_2 production following treatment with the two extracts tested was similar between 24 and 48 h (Fig. 4). Treatment with the dichloromethane extract at 500 μg/ml for 48 h showed 43.5 % inhibition of PGE_2 production, but an opposite effect with 1000 μg/ml: stimulation of synthesis by about 318 %. Consequently, this extract seemed to be pro-inflammatory via cyclooxygenases at high concentrations. Treatment with the ethyl acetate extract showed dose dependent stimulation of PGE_2 production, reflecting the property of this extract.

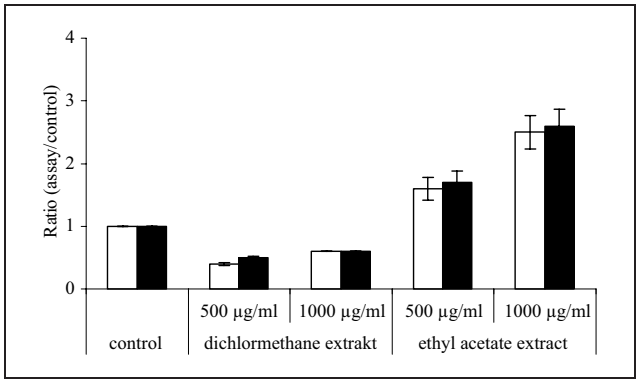

Fig. 3: Quantification of DNA fragmentation after *Daphne gnidium* L. extract treatment. Cells treated or not (control) for 24 (□) and 48h (■) with the indicated concentrations. Apoptosis quantified on floating and adherent cells using "cell death" ELISA kit (Cell Death Detection ELISA^{Plus}, Roche Diagnostics). Apoptotic ratio was determined as sample absorbance/control absorbance. Values expressed as mean ± SD of three experiments

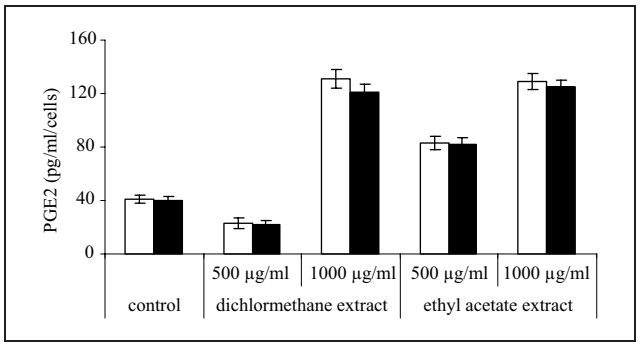

Fig. 4: Effect of *Daphne gnidium* L. extracts on PGE_2 release. Cells treated or not (control) for 24 (□) and 48h (■) with the indicated concentrations. Amounts of PGE_2 released by cell monolayers in culture supernatants quantified by EIA. PGE_2 levels normalized to number of adherent cells. Values expressed as mean ± SD of three experiments (p-value relative to control group: $p < 0.05$)

3. Discussion

The present study showed the antiproliferative effect of the hexane, dichloromethane and ethyl acetate extracts of *Daphne gnidium* L. on the MCF-7 breast cancer cell line. The difference of the results between the four extracts is normal since every solvent extract differs, comprising a set of products of different chemical structure according to their polarity and affinity. The comparison of IC_{50} values of the three active extracts seemed to indicate that the compound responsible for the antiproliferative effect was concentrated in the dichloromethane extract. By comparing the IC_{50} of the dichloromethane extract with those of other crude extracts of plants with anti-cancer activity (Liang et al. 2002; Ruffa et al. 2002), it appeared that *Daphne gnidium* L. inhibits strongly the growth of MCF-7 cells. Our results were in agreement with a previous study showing that another Chinese plant of the same botanical family, non identical ecologically and of a different species to the one we studied, named *Daphne genkwa* exhibited an anti-neoplastic effect on the P-388 and A-549 cell lines (Liou et al. 1982; Zhan et al. 2005).

The study of the morphology of MCF-7 cells treated with the three active extracts indicated the cytotoxicity of the hexane extract as opposed to two others. This result was quantitatively confirmed by the LDH test.

Apopotosis is believed to be one of the major consequences of anticancer drug treatment against malignancies. Our study showed a dose dependent pro-apoptotic effect of the ethyl acetate extract, whereas the results for apoptosis induction by the dichloromethane extract were inconclusive. As regards the results for the dichloromethane extract, microscopic observations seemed to indicate onset of apoptosis following treatment as assessed by apoptotic features such as nuclear condensation and membrane blebbing. Nevertheless, quantification of apoptosis at 24 and 48 h of dichloromethane extract treatment was not in agreement with these observations. So it is uncertain whether at these times, apoptosis was complete and DNA fragments were no longer detectable, or the antiproliferative effect of this extract involved another mechanism such as cell cycle arrest.

Furthermore, results from the PGE_2 production assay indicated that the ethyl acetate extract and the dichloromethane extract at high concentration (1000 µg/ml) stimulated PGE_2 release from treated cells. In addition, the dose dependent increase of PGE_2 synthesis induced by the ethyl acetate extract followed the same profile as that of apoptosis induction. From this two assumptions could arise: the increase in the production of PGE_2 was involved in the induction of apoptosis or the overproduction of PGE_2 was a response to imposed cellular stress. Indeed, Hong et al. (2002) showed that *Daphne genkwa* was considered to be a potential inhibitor of nitric oxide synthase, but not an inhibitor of COX-2 activity, which agrees with our results for *Daphne gnidium* L.

Furthermore, increased COX-2 expression and increased PGE_2 production associated with apoptosis induction have been reported with several natural products such as diosgenin (Leger et al. 2004; Liagre et al. 2004) or areca nut ingredients (Chang et al. 2004).

In conclusion, our study suggested that *Daphne gnidium* L. must be carefully considered when used as a chemopreventive agent. Future work in our laboratory will identify and characterize the active compounds present in the most promising extract, following fractionation using advanced techniques for fractioning and identification.

4. Experimental

4.1. Plant material

Daphne gnidium L. was collected during March 2004 from its natural location in Morocco and taxonomically identified at the National Institute of Medicinal and Aromatic Plants, Taounate. A voucher specimen (FMP-49) is deposited at the herbarium of the Faculty of Medicine and Pharmacy, Rabat.

4.2. Plant extract

The dried roots were crushed, and 100 g of powder were extracted on soxhlet over at least 48 h by four organic solvents with increasing polarity: hexane, dichloromethane, ethyl acetate and methanol.

The recovered extracts were concentrated by the rota-vapor and were stored at −20 °C until analysis.

4.3. Cell line, cell culture and treatment

Human breast tumor cells (MCF-7) were seeded at density of 6×10^4 cells/ml in 75 cm^2 tissue culture flasks, grown in Dulbecco's modified Eagle's medium (Gibco BRL, Cergy-Pontoise, France) supplemented with 10% fetal bovine serum (Gibco BRL), 1% L-glutamine and 1% penicillin/streptomycin mixture (Gibco BRL). Cultures were maintained in a humified atmosphere with 5% CO_2 at 37 °C to subconfluence. Fresh medium was supplied every 48 h. The doubling time of MCF-7 cells was estimated to be 18 h by growth curve studies.

Cells were allowed to grow for 24 h in culture medium prior to exposure to *Daphne gnidium* L. extracts for 24, 48 and 72 h. A stock solution of 50 mg/ml of extracts was prepared in dimethylsulfoxide (DMSO) (Sigma) and diluted in culture medium to give a final concentration of 1 to 1000 µg/ml. The same amount of DMSO was added to control cells.

4.4. Cell proliferation assay

Medium was aspirated from MCF-7 cells grown to about 90% confluence. Cells were washed with PBS, trypsinized, counted with a hemocytometer and subcultured into 96-well plates with 6×10^3 cells per well in 100 µl medium. After 24 h incubation at 37 °C in a 5% CO_2 incubator, the seeding medium was removed and replaced by *Daphne gnidium* L. extracts diluted in medium to a final concentration of 1 to 1000 µg/ml. Measurement of cell proliferation was determined using the MTT assay as previously described (Moalic et al. 2000) and experiments were performed in sextuple assays. The absorbance was read at a wavelength of 550 nm using a microtiter plate reader (Multiskan EX, Labsystems). Parallel to the MTT test, we studied the change of morphology of MCF-7 cells treated with the *Daphne gnidium* L. extracts.

4.5. Determination of IC_{50} concentration of Daphne extracts

The absorbance values obtained per treatment were converted to percentage proliferation. Regression analysis was performed on MTT assay proliferation data and the resulting equation used to compute IC_{50}.

4.6. Lactate deshydrogenase test

Cells were seeded in 96-well plates at a density of 6×10^3 cells/well and treated without or with *Daphne gnidium* L extracts. A cytotoxicity detection kit (Boehringer Mannheim) measured the LDH activity released from the cytosol of damaged cells into the supernatant to evaluate the percentage of lytic cell death compared to the maximum cell lysis obtained by Triton X-100 treatment according to the manufacturer's protocol.

4.7. Quantification of apoptosis

Apoptotic cell death was quantified by "cell death" ELISA (Cell Death Detection ELISA^{Plus}, Roche Diagnostics) as described elsewhere (Moalic et al. 2001). Briefly, MCF-7 cells were cultured in 75 cm^2 tissue culture flasks. After treatment with *Daphne gnidium* L. extracts, cell lysates were obtained from pooled floating and adherent cells, according to the manufacturer's protocol. Apoptosis from control and treated cells was then measured as previously described (Corbiere et al. 2003).

4.8. PGE_2 EIA analysis

MCF-7 cells were cultured and treated (24 and 48 h) as described above in 75 cm^2 tissue culture flasks. Undiluted culture supernatants were centrifuged (2000 rpm for 5 min at 4 °C) before being stored at −80 °C until analysis. PGE_2 release by cell monolayers was measured by PGE_2 competitive immunoassay (Cayman Chemicals) carried out as previously described (Liagre et al. 2004) according to the manufacturer's protocol. PGE_2 production was normalized with respect to the number of viable cells present in the particular culture at the time of sampling.

4.9. Statistical analysis

The median and standard deviation (SD) were calculated using Excel (Microsoft Office, Version 2003). Statistical analysis of differences was carried out by analysis of variance (ANOVA). A P-value of less than 0.05 was considered to indicate significance.

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