

Antitumoral activity of *Ficus carica* L. on colorectal cancer cell lines

Hala Soltana¹, Aline Pinon², Youness Limami^{3*}, Younes Zaid^{3,5}, Loubna Khalki⁴, Nabil Zaid⁵, Driss Salah⁵, Uteuliyev Yerzhan Sabitaliyevich⁶, Alain Simon², Bertrand Liagre², Mohamed Hammami¹

¹ Biochemistry Laboratory, LR12ES05 "Nutrition- Functional Foods and vascular Health", Faculty of Medicine, University of Monastir, Monastir, Tunisia

² Laboratory of Chemistry of Natural Substances, Faculty of Pharmacy, University of Limoges, FR 3503 GEIST, EA 1069, Limoges, France

³ Research Center of Abulcasis University of Health Sciences, Rabat, Morocco

⁴ Research Center, Mohammed VI University for Health Sciences, Casablanca, Morocco

⁵ Department of biology, Faculty of Sciences, Mohammed V University, Rabat, Morocco

⁶ Kazakhstan Medical University "KSPH", Kazakhstan

*Correspondence to: limami@hotmail.com

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Abstract: In traditional medicine, *Ficus carica* (also known as fig) latex is recognized as a remedy with various therapeutic effects. In the present study we investigated the antitumor activity of *Ficus carica* extracts and latex. We evaluated the effects of increasing concentrations of *Ficus carica* extracts and latex on HCT-116 and HT-29 human colorectal cell proliferation using MTT assay and apoptosis induction by evaluating PARP cleavage by Western blot analysis. Peel, pulp, leaves, whole fruit and latex extracts of *Ficus carica* exerted significant antiproliferative effects on HCT-116 (IC₅₀ values 239, 343, 177, 299, 206 µg/ml) and HT-29 cells (IC₅₀ values 207, 249, 230, 261, 182 µg/ml) after 48h of treatment. Furthermore, treatment with different extracts of *Ficus carica* induced apoptosis in both HT-29 and HCT-116 cancer cells. Leaves and latex extracts of *Ficus carica* showed the strongest antiproliferative activities. Overall, our results showed that these natural products are strong apoptosis inducers which suggest their use of for therapeutic purposes.

Key words: Natural Products; Apoptosis Inducers; Colorectal Cancer; *Ficus carica*.

Introduction

Today, with more than 700.000 deaths a year, colorectal cancer is one of the most common types of cancer and the fourth leading cause of cancer deaths in the world (1). Westernized diet and lifestyle are believed to be the most influent factors to increasing colorectal cancer incidence worldwide. Colorectal cancer treatment (chemotherapy) suffers from chemoresistance and toxicity which alters treatment efficiency. Natural products and their molecular frameworks provide valuable starting points for drug discovery and medicinal chemistry. Natural products researchers and pharmacologists have started to investigate medicinal value of wide ranging bioactive molecules from natural sources. More importantly, bioactive chemicals from different plants have been shown to efficiently modulate signaling pathways and apoptosis in different cancers (2-12).

Indeed, natural products are generally used as alternative treatment for various diseases and considered to have minor side effects (13-16). Since ancient times, *Ficus carica* L. (Moraceae) has been used traditionally as a medicinal fig tree by various cultures. Fig fruit (*Ficus carica* L.) contains high level of polyphenols, flavonoids, anthocyanins and exhibited high anti-oxidant capacity (17). Consumers eat figs in different forms; dried, fresh, as a jam or as a juice. Generally, they peel

figs; eat the pulp and discard the peel (18). However, in some countries, people prefer to eat the whole fruit (19) and its by-products (leaves, latex, bark and roots). Several *in vivo* and *in vitro* studies showed that these by-products can be used as treatment in various disorders such as gastrointestinal, respiratory, inflammatory, cardiovascular disorders and cancers (20, 21). Rubnov *et. al.*, showed that the Fig latex has an antiproliferative activity in several cancer cell lines (22). In another study, Fig latex has been shown to have antitumor activity in xenografted and spontaneous tumors in mice (23, 24). However, antitumor activity of *Ficus carica* against colorectal cancer cells remains to be investigated.

In the present work, we have evaluated for the first time the antiproliferative and pro-apoptotic effects of ethyl acetate extract from *Ficus carica* peel, pulp, leaves, whole fruit and latex on HCT-116 and HT-29 human colorectal cancer cells. HCT-116 and HT-29 are cell lines that represent different stage of colorectal cancer. Furthermore, while HT-29 have mutant form of tumor suppressor p53, HCT-116 have wild type one. As p53, is described to play a key role in chemoresistance these two cell lines represent good models to study their responsiveness to *Ficus carica* extracts and then determine whether active principles from Fig fruit could be used in colorectal cancer treatment (25).

Materials and Methods

Plant identification

An agricultural technician performed identification of fruit variety used. It's Kholi variety. Kholi variety is a dark type; black peel with dark red pulp. Fruits are sweet and juicy. No voucher specimen of this variety has been deposited in publicly herbarium.

Sample collection

Latex, fig fruits and leaves (*Ficus carica*) of variety Kholi were collected at full maturity from region Mahdia located at the Central East of Tunisia (Altitude 7m; Latitude: 35°30'16"N, Longitude: 11°03'43"E) (19). The Kholi variety used for this research is a cultivated variety. It belongs for a smyrna type that requires pollinisation for development of good quality fruit. This collection of samples was authorized by the Regional Commissariat for Agricultural Development of Mahdia, Tunisia (CRDA).

Latex, leaves and fruits were placed in a 4°C refrigerated box and transported within 3h to the Herbarium of the laboratory of Biochemistry, Faculty of Medicine, Tunisia.

Preparation of fig latex

Fig latex was collected dropwise from ripe fig fruit (*Ficus carica*, in Mahdia, Tunisia) without squeezing and kept at -20°C as stock solution (1 mg/ml). Then, serial dilutions using dimethyl sulfoxide as vehicle were realized to prepare solutions of concentrations from 10 to 800 µg/ml.

Preparation of leaves, fruits, peels and pulps of *Ficus carica*

Leaves were sampled at their fully mature stage at last week of august from Mahdia. They were washed and air-dried until constant weight, then ground (Moulinex, France). Ground preparation was sieved, and the fine powder obtained was transferred to darkness for further use. After washing the fresh fruits with deionized water, they were peeled to separate peel and pulp. Each part of fruit was lyophilized and ground into fine particles. Then, 1 g of the ground sample was extracted with 10 ml of ethyl acetate at room temperature for 24 h in a shaking water bath. The mixture was centrifuged at 4200 g for 30 min, and the supernatant was collected. Ethyl acetate in the crude extract was removed under vacuum using a rotary evaporator at 50°C. The extract was dissolved in dimethyl sulfoxide to obtain 1 mg/ml stock solution and stored at 4°C for further use.

Cell lines, cell culture and treatment

HCT-116 (ATCC® CCL-247™) and HT-29 (ATCC® HTB-38™) human colorectal cell lines were purchased from American Culture Type Collection (LGC Standards, Middlesex, UK). Cells were seeded at 3×10^4 /cm² and grown in RPMI 1640 or DMEM (Gibco BRL, Cergy-Pontoise, France) supplemented with 10% fetal calf serum (FCS) (Gibco BRL), 1% L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco BRL) for HCT-116 and HT-29 respectively. Cultures were maintained in a humidified atmosphere with 5% CO₂ at 37°C. Cells were allowed to grow for 24 h in

culture medium and then treated or not with extracts of peel, pulp, leaves, whole fruit and latex of *Ficus carica* at indicated concentrations and times. Of note that the same amount of vehicle (DMSO) was added to control cells with a percentage of DMSO not exceeding 0.3%.

Cell proliferation assay

The effects of fig extracts on the viability of HCT-116 and HT-29 cells were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (26). After treatment with fig extracts or DMSO for 24 h and 48 h, 10 µl of 5 mg/ml MTT was added to each well and incubated for 4h. The supernatant was removed and 100 µl DMSO was added per well. After shaking samples for 5 min, the absorbance was measured at 550 nm in a microplate reader (Bio-Rad, Marnes-la-Coquette, France), using wells without cells as blank. All experiments were performed in sextuple assays. The effect of peel, pulp, leaves, whole fruit and latex of *Ficus carica* on the proliferation of cancer cells was expressed as relatively cell viability, using the following formula: Percent viability = (OD of drug-treated sample/OD of none treated sample) × 100 (27). The IC₅₀ value was the concentration of extract which inhibited cell growth by 50% compared to controls.

Protein extraction and western blot analysis

For total protein extraction, cells were collected, washed with PBS and homogenized in RIPA lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40, 10% SDS, 20 µg/ml of aprotinin) (Sigma, St Quentin Fallavier, France) containing protease inhibitors (Complete™ Mini, Roche Diagnostics, Meylan, France) according to the manufacturer's protocol.

Western blot analysis was performed as described previously (28).

Statistical analyses

All results are presented as means ± SD of at least three replications (at least three individual extract per each fruit were used) and statistical analyses were performed using the statistical Package for Social Sciences (SPSS) program, release 16.0 for Windows (SPSS, Chicago, IL, USA). Measurements of percentage of living cells were compared to control via an independent Man Whitney U Test. IC₅₀ was determined by one-way ANOVA using Tukey's test. Statistical significance was defined to be at a level of $p < 0.05$.

Results

Ethyl acetate extracts of *Ficus carica* reduce HCT-116 and HT-29 cell proliferation

Cells were cultured with or without ethyl acetate extract of peel, pulp, leaves, whole fruit and latex of *Ficus carica* for 24 and 48h, and relative cell proliferation was assessed by MTT assay with (10-800 µg/ml) *Ficus carica*'s ethyl acetate extract treatment for HCT-116 (Fig.1A) and HT-29 (Fig.1B). Then, the percentage of relative cell viability of treated cells compared to control was determined.

As shown in figure 1, a decrease in proliferation was observed after 24h and 48h of incubation with differ-

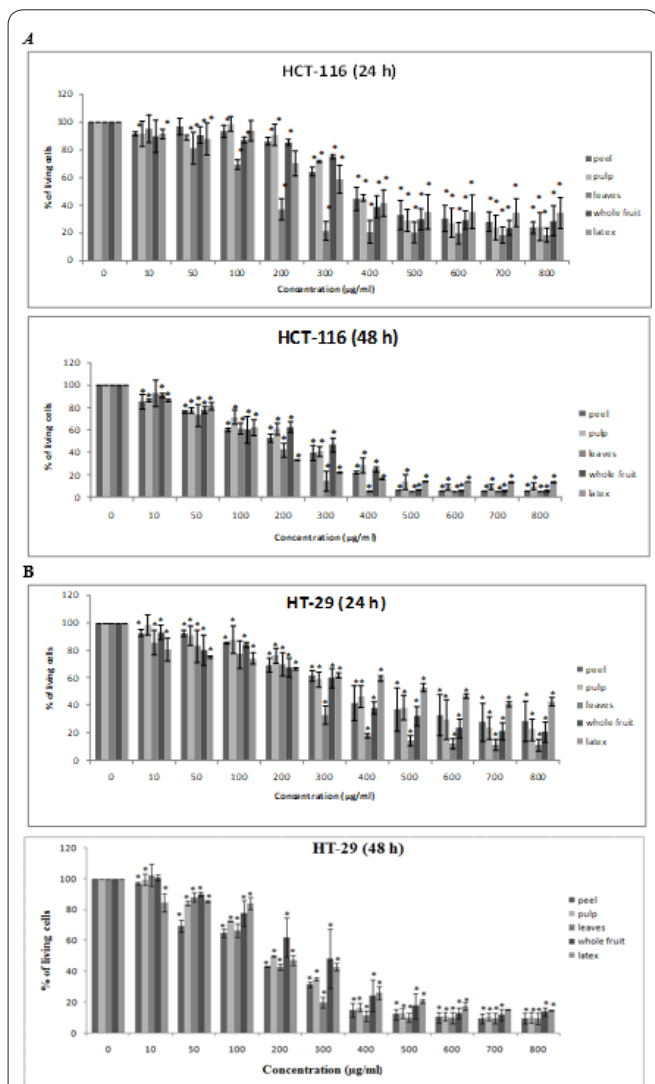


Figure 1. Growth inhibition of extracts of peel, pulp, leaves, whole fruit and latex on HCT-116 and HT-29 cells. Cells were seeded onto 96-well plate at 1×10^4 /well and were treated with extracts at different concentrations and percentage of cell viability was determined by MTT assay after 24 and 48h of treatment. A dose dependent growth inhibition was observed at concentrations ranging from 10 to 800 $\mu\text{g/ml}$. Results are mean values \pm SD of three independent experiments performed in sextuple assays. Growth inhibition of extracts of fig on human HCT-116 cells (A). Growth inhibition of extracts of fig on human HT-29 cells (B). Results are presented as percentage of living cells compared to control ($n=3$, $* p < 0.05$).

ent extracts of peel, pulp, leaves, whole fruit and latex of *Ficus carica* in a dose- dependent manner both in HCT-116 and HT-29 cells. This antiproliferative effect of the ethyl acetate extracts of *Ficus carica* was more important in HCT-116 rather than HT-29 cells especially after 24h of treatment (peel: 33.04%, pulp: 29.13%,

leaves: 20.54%, whole fruit: 29.83% and latex: 35.25% of living cells vs. peel: 37.45%, pulp: 38.72%, leaves: 14.69%, whole fruit: 32.62% and latex: 53.46% of living cells, for HCT-116 and HT-29 cells respectively, when treated with 500 $\mu\text{g/ml}$ of ethyl acetate extract). The effects of *Ficus carica* extracts on proliferation of both HCT-116 and HT-29 cell lines seem to be more effective on HCT-116.

Furthermore, the leaves extracts appeared the most susceptible to inhibit the growth of HCT-116 and HT-29 colon cancer cells than other extracts of *Ficus carica* studied. Indeed, it had the highest antiproliferative capacity at their lowest concentrations tested.

The antiproliferative response obtained with different extracts of *Ficus carica* (peel, pulp, leaves, whole fruit and latex) is shown in Table 1 with IC_{50} values for each extract. The five tested peel, pulp, leaves, whole fruit and latex extracts of *Ficus carica* presented potent inhibitory activity on HT-29 cell growth in a dose dependent manner with IC_{50} values of: leaves (260 and 230 $\mu\text{g/ml}$), whole fruit (381 and 261 $\mu\text{g/ml}$), pulp (416 and 249 $\mu\text{g/ml}$), peel (467 and 207 $\mu\text{g/ml}$) and latex (503 and 182 $\mu\text{g/ml}$) after 24 and 48 h respectively. MTT assay determined that the following extracts were also highly active against HCT-116 colon cancer cells: leaves (320 and 177 $\mu\text{g/ml}$), whole fruit (391 and 299 $\mu\text{g/ml}$), pulp (399 and 343 $\mu\text{g/ml}$), latex (402 and 206 $\mu\text{g/ml}$) and peel (432 and 239 $\mu\text{g/ml}$) after 24 and 48 h respectively. As shown with experiments results the leaves presented the highest antiproliferative activity both for HCT-116 and HT-29 cells. Furthermore, we also showed that latex had a higher antiproliferative activity than other extracts of *Ficus carica* after 48h of treatment.

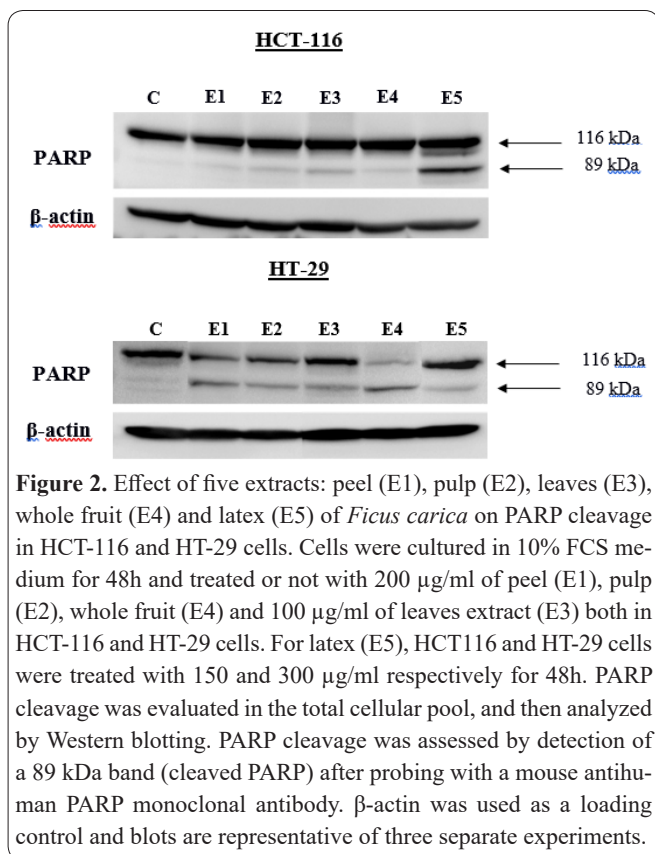
Apoptosis induction by *Ficus carica* extracts

Western blot analysis using poly (ADP-ribose) polymerase (PARP) monoclonal antibody showed that ethyl acetate extracts of *Ficus carica* induced an increase in the expression level of cleaved PARP (Fig. 2) in both cell lines. Our results showed a dominant effect of five ethyl acetate extracts of *Ficus carica* on HT-29 cells compared to HCT-116 cells. Indeed, in HT-29 cells, the five extracts (peel, pulp, leaves, whole fruit and latex) of *Ficus carica* have a decrease in the expression level of the PARP native form (116 kDa), that is more or less important depending on each extract, with the appearance of the cleaved form (89 kDa), which is one of the hallmarks of apoptosis. In HCT-116 cells, the expression of the native form does not vary relative to the control. Nevertheless, there is occurrence of the cleaved form in different extracts that appeared more pronounced in the latex extract.

Table 1. IC_{50} values of the extracts of fig for HCT-116 and HT-29 cells.

IC_{50}	HCT-116		HT-29	
	24h	48h	24h	48h
Peel	432 \pm 84.73a	239 \pm 39.07ab	467 \pm 40.96b	207 \pm 45.78a
Pulp	399 \pm 10.42a	343 \pm 40.25b	416 \pm 45b	249 \pm 43.19a
Leaves	320 \pm 39.75a	177 \pm 26.58a	260 \pm 21.17a	230 \pm 47.35a
Whole fruit	391 \pm 2.23a	299 \pm 18.38ab	381 \pm 31.07ab	261 \pm 27.9a
Latex	402 \pm 77.76a	205,71 \pm 55.71ab	503 \pm 52.5b	182 \pm 7a

The results were expressed as mean \pm SD. $n = 3$. Values with no letters in common are significantly different ($p < 0.05$).



Discussion

In the present study, we investigated the potential antitumor activity of *Ficus carica* extracts and latex on HCT-116 and HT-29 human colorectal cancer cells.

We evaluated the effects of increasing concentrations of *Ficus carica* extracts and on cell proliferation. Results document a great antiproliferative activity of the different extracts and latex with a higher activity for leaves extracts and latex.

Khodarahmi *et al.* (2011) showed that fruits, leaves and latex of *Ficus carica* have cytotoxicity against HeLa cell line with approximate IC_{50} values of 10-20 µg/ml (29). In another study, it has been shown that plant extracts have the ability to significantly decrease the cell viability in a time- and dose-dependent manner (28). Rubnov *et al.* showed that the Fig latex has an antiproliferative activity in several cancer cell lines (22). *In vivo* studies performed by Ullman *et al.* showed that fig fresh latex has antitumor activity in xenografted and spontaneous tumors in mice (23, 24).

Our results also document that the antiproliferative activity was higher in HCT-116 compared to HT-29. We could hypothesize that the difference in susceptibility between HT-29 and HCT-116 could be due to the difference in ethyl acetate composition (30, 31). Furthermore, it has been shown that HT-29 is a highly aggressive cell line compared to HCT-116 and the difference in invasive competence between the two cell lines may account for their responsiveness to treatment (32, 33).

Then, by evaluating PARP cleavage, we showed that Fig extracts and latex were able to induce apoptosis in both HT-29 and HCT-116 colorectal cancer cells.

PARP plays a key role in base excision repair mechanism of single-strand DNA breaks in cells. During apoptosis, especially at late stage, PARP is cleaved and

inactivated by caspase-3 (34, 35). With the executioner caspase-3, PARP represents one of the most important apoptosis markers. Indeed, its inhibition prevents cancer cells from apoptosis induction (36). Several studies reported the key role of PARP in apoptotic process. Yoon *et al.*, showed that apoptosis induction by tanshinone IIA in HL60 leukemic cells was accompanied by caspase-3 activation and subsequent proteolytic cleavage of PARP (37). Other *in vitro* and *in vivo* studies performed by Wang *et al.*, also showed an important role played by PARP in apoptosis induction (38, 39).

Overall, our results showed a great antiproliferative and pro-apoptotic activity of *Ficus carica* extracts on both HT-29 and HCT-116 colorectal cancer cells. Taken together, our results, as well as results from previous studies document that Fig fruit have great antitumor activity. The anticancer activity associated with their antioxidant properties and their rich composition of bioactive compounds (40) make them great candidates to be used as chemotherapeutic or chemopreventive agents (41). Further experiments are in progress to understand the molecular targets and pathways affected by *Ficus carica*.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author's Contributions

SH, PA and YL, LK, NZ designed and performed the majority of experiments, analyzed data, and assisted with writing the manuscript. UYS, AS, YZ, BL and MH analyzed data, and prepared the manuscript. All authors read and approved the final manuscript.

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