Effects of an extract of *Celtis aetnensis* (Tornab.) Strobl twigs on human colon cancer cell cultures

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Abstract. Cancers of the digestive tract, in particular colorectal cancer (CRC), are among those most responsive to dietary modification. Research has shown that approximately 75% of all sporadic cases of CRC are directly influenced by diet. Many natural compounds have been investigated for their potential usefulness as cancer chemopreventive agents as they have been thought to suppress carcinogenesis mainly during the initiation phase due to their radical scavenger activity. Since there is an increasing interest in the *in vivo* protective effects of natural compounds contained in plants against oxidative damage involved in several human diseases such as cancer, the aim of the present research was to test the effects of a *Celtis aetnensis* (Tornab.) Strobl twig extract on a human colon carcinoma cell line (Caco2). In order to elucidate the mechanisms of action of this extract, LDH release, GSH content, ROS levels, caspase-3 and γ-GCS expression were also evaluated. The results revealed that the *Celtis aetnensis* extract reduced the cell viability of the Caco2 cells inducing apoptosis at the lowest concentration and necrosis at higher dosages. In addition, this extract caused an increase in the levels of ROS, a decrease in RSH levels and in the expression of HO-1. The expression of γ-GCS was not modified in the *Celtis aetnensis*-treated Caco-2 cells. These results suggest an interference of this extract on the oxidant/antioxidant cell balance with consequent cell damage. The present study supports the growing body of data suggesting the bioactivities of *Celtis aetnensis* (Tornab.) Strobl and its potential impact on cancer therapy and on human health.

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In Memoriam: This work is dedicated to the memory of Professor Liliana Iauk.

Introduction

Colorectal cancer is the third most common malignancy in males and the second most common in females, with significant variations in the worldwide distribution. In veterinary medicine it was described in sheep and dog with a different incidence (1). A more than 10-fold variation in the colorectal cancer incidence rate across countries and the rapid increase in incidence rates in countries experiencing industrialization suggest a strong link with lifestyle factors. The highest incidence rates are found in economically developed countries, whereas the lowest rates are noted in Africa and South-Central Asia (2). However, recent 'perturbations' in colorectal cancer incidence trends were observed probably resulting from a combination of risk factors, including obesity, sedentary lifestyle, increased prevalence of smoking, excessive alcohol consumption and 'westernization' in dietary habits - a diet rich in red and processed meat and low intake of fruits and vegetables (3,4).

The possibility that fruit and vegetables may help to reduce the risk for various types of cancer raised great interest already in the 1970s, when studies conducted to assess differences in cancer rates and diet between countries, suggested that various dietary factors may have important effects on cancer risk (5,6). Several years later, a joint report by the World Cancer Research Fund together with the American Institute of Cancer Research found 'convincing' evidence that a high fruit and vegetable intake would reduce cancer of the colon and rectum (4,7).

It is known that plants may be used both as a medicine and a food and it is difficult to draw a line between these two groups: food may be medicine, and *vice versa*. In traditional societies, plants are often used multi-contextually, for example, as food and for medicine (8). Particularly interesting are the pharmacological properties of these plants and of the constituents isolated from them.

Epidemiologic studies suggest that the consumption of natural compounds lowers the risk of cardiovascular disease, diabetes, arthritis and cancer (4,9).

The genus *Celtis* (Ulmaceae) includes about 70 species of shrubs or trees, primarily distributed in the temperate and tropical regions of the Northern Hemisphere (10). *Celtis aetnensis* (Tornab.) Strobl is a bushy shrub present on Mount Etna (Sicily) (11). It is a woody plant, whose leaves are heart-shaped and slightly crenate on the edge. Some species of *Celtis* are used in traditional medicine for the treatment of low back pain, upset stomach, abdominal pain, as well as for their astringent and soothing in case of diarrhea, enteritis and inflammation of the oral cavity. These properties are primarily attributable to the active principles contained in the leaves, such as tannins, saponins and flavonoids.

In previous phytochemical reports on species of the genus *Celtis*, the presence of betulin, gallic acid, 3,3'-di-O-methyl-lellagic acid, moretenol and two triterpene esters, 3β-*trans*-sinapoyloxylup-20(29)-en-28-ol and 3β-*trans*-feruloyloxy-16β-hydroxylup-20(29)-ene and five known triterpenes, 3β-O-(E)-feruloylbetulin, 3β-O-(E)-coumaroylbetulin, betulin, 20-epibryonolic acid, and ursolic acid, were isolated from the twigs of *C. philippinensis* (10,12).

Antioxidant and cytotoxic activities were reported for other *Celtis* species e.g., *Celtis philippinensis* Blanco, *Celtis africana* Burm. f., and *Celtis iguanae* (Jacq.) Sarg. (10,13-16), however, as far as we know, no previous biological and phytochemical investigations on *Celtis aetnensis* (Tornab.) Strobl have been reported.

Many antioxidant compounds have been investigated for their potential usefulness as cancer chemopreventive agents (17-19). Since there is an increasing interest in the *in vivo* protective effects of natural compounds contained in plants against oxidative damage involved in several human diseases such as cancer, the present study investigated the effects of *Celtis aetnensis* (Tornab.) Strobl twig extract on the viability of the human carcinoma Caco2 cell line. In addition, in order to elucidate the mechanisms of action of this extract, LDH release, caspase expression, thiol groups, ROS levels, HO-1 and γ-GCS protein expression were also evaluated.

Materials and methods

Chemicals. 3-(4,5-Dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were obtained from Sigma Aldrich Co. (St. Louis, MO, USA). All other chemicals were purchased from Gibco-BRL Life Technologies (Grand Island, NY, USA). Polyclonal γ-glutamylcysteine synthetase (γ-GCS) and caspase antibodies were from Abcam (Victoria, BC, Canada). Secondary horseradish peroxidase-conjugated anti-rabbit antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The enhanced chemiluminescence system for developing immunoblots and nitrocellulose membranes was purchased from Amersham (Milano, Italy). The ELISA kit, used to measure heme oxygenase-1 (HO-1) protein concentration, was from Stressgen Biotechnologies (Victoria, BC, Canada).

Plant material and preparation of the extract. Twigs of *Celtis aetnensis* (Tornab.) Strobl (Ulmaceae) were collected in the area around Linguaglossa (Catania, Italy) in June 2015. The specimens were obtained thanks to the Regional Forest Corps Detachment of Catania-Nicolosi and authenticated by botanist Professor S. Ragusa, Department of Health Sciences, University of Catanzaro (Italy). A voucher specimen of the plant was deposited in the herbarium of the same department.

Twigs of *Celtis aetnensis* (Tornab.) Strobl were air dried for 10 days, and then washed free from soil, powdered and stored in airtight containers at room temperature until extraction. One fraction of 50 g of the powdered twigs was exhaustively extracted by maceration with MeOH (w/v ratio of 1:5) at room temperature for 48 h. The filtrate was dried under vacuum using a rotary evaporator, and the residue that dissolved in water was extracted with hexane. The aqueous solution was further extracted with chloroform. Finally the chloroform solution was brought to dryness (residual weight 133.6 mg).

Cell culture and treatments. Human colon carcinoma cells (Caco2), obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA), were cultured in Dulbecco's modified essential medium (Gibco-BRL Life Technologies) supplemented with 10% foetal calf serum, 1 mmol/l sodium pyruvate, 2 mmol/l L-glutamine, streptomycin (50 mg/ml) and penicillin (50 U/ml).

The cells were plated at a constant density to obtain identical experimental conditions in the different tests, thus to achieve a high accuracy of the measurements. After a 24-h incubation at 37˚C under a humidified 5% carbon dioxide atmosphere to allow cell attachment, the cells were treated with different concentrations of the chloroformic extract of twigs from *Celtis aetnensis* (Tornab.) Strobl (5, 50, 100, 250 or 500 μg/ml), and incubated for 72 h under the same conditions. Chloroformic extract was dissolved in dimethyl sulfoxide (DMSO) and diluited with medium to give final concentrations of total extract ranging from 5 to 500 μg/ml. Four replicates were performed for each sample.

At the end of the treatment, the cells were scraped, washed with PBS and immediately utilized for analysis.

MTT bioassay. MTT assay was performed to monitor cell viability, measuring the conversion of tetrazolium salt to yield colored formazan, the amount of which is proportional to the number of living cells. For this assay, the cells were set up (8x10^3 cells/well) in a 96-multiwell flat-bottomed 200 μl microplate (20). The optical density of each well was measured with a microplate spectrophotometer reader (Titertek Multiskan; Flow Laboratories, Helsinki, Finland) at λ=570 nm.

Lactic dehydrogenase release. Lactic dehydrogenase (LDH) release was measured to evaluate cell necrosis as a result of cell membrane disruption. LDH activity was measured spectrophotometrically in the culture medium and in the cellular lysates, at λ=340 nm by analyzing NADH reduction (21). The percentage of LDH release was calculated as the percentage of the total amount, considered as the sum of the enzymatic activity present in the cellular lysate and that in the culture medium.

Thiol group determination. Thiol groups (RSH) were measured using a spectrophotometric assay as previously described (22).
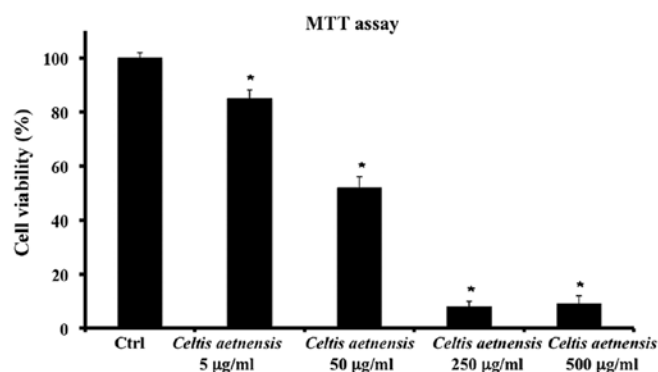
Figure 1. Cell viability in the Caco2 cells untreated and treated for 72 h with a chloroformic extract of *Celtis aetnensis* (Tornab.) Strobl at different concentrations (5-500 µg/ml). Values are the mean ± SD of four experiments in triplicate. Significant vs. untreated control cells, *P<0.001.
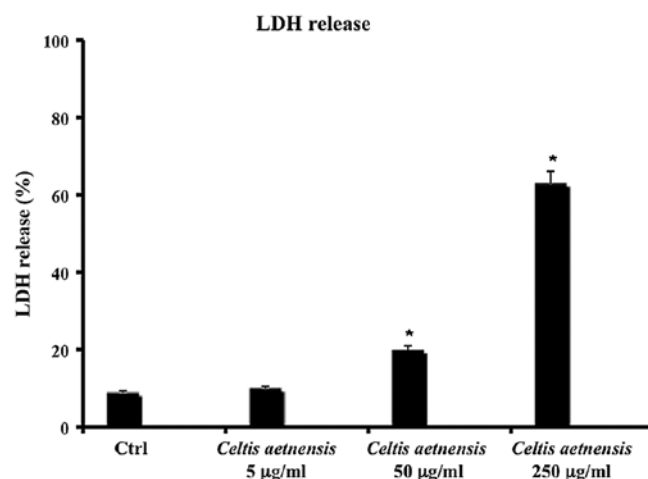

Figure 2. LDH released in the Caco2 cells untreated and treated for 72 h with a chloroformic extract of *Celtis aetnensis* (Tornab.) Strobl at different concentrations (5-250 µg/ml). Values are the mean ± SD of four experiments in triplicate. Significant vs. untreated control cells, *P<0.001.

Results are expressed as µmol/mg protein. Protein concentration was measured according to Bradford (23).

Reactive oxygen species assay. Determination of reactive oxygen species (ROS) was performed using a fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA), as previously described (24). The fluorescence (corresponding to the oxidized radical species 2',7'-dichlorofluorescein, DCF) was monitored spectrofluorometrically (excitation, λ=488 nm; emission, λ=525 nm). The total protein content was evaluated for each sample, and the results are reported as the percentage increase in fluorescence intensity/mg protein with respect to the control (untreated) cells.

HO-1 protein expression. A commercially available enzyme-linked immunosorbent assay (ELISA) kit was used to measure HO-1 protein concentration in the cellular lysates. The assay was performed in accordance with the protocol provided by the manufacturer. Absorbance at λ=450 nm was measured and HO-1 concentration was calculated from a standard curve generated with purified HO-1 (25). The limits of detection provided by the manufacturer were 0.78-25 ng/ml. Results are expressed as ng/mg protein.

Western blottings. Caco2 cells were harvested using cell lysis buffer. The lysate was collected for western blot analysis and protein levels were visualized by immunoblotting with antibodies against γ-GCS and caspase-3 as previously described (21).

Statistical analysis. One-way analysis of variance (ANOVA) followed by Bonferroni's t-test was performed in order to estimate significant differences among groups. Data are reported as mean values ± SD, and differences between groups were considered to be significant at P<0.005.

Results

Effects of Celtis aetnensis (Tornab.) Strobl on Caco2 cell viability. The treatment of Caco2 cells with 5, 50, 250 or 500 µg/ml of chloroformic extract of *Celtis aetnensis* (Tornab.)

Strobl for 72 h resulted in a significant and dose-dependent reduction in cell viability (Fig. 1); since 250 and 500 µg/ml of chloroformic extract showed similar effects in other experiments we used 250 µg/ml of the extract.

LDH release. Necrosis results in distruption of the cytoplasmic membrane with release of cytoplasmic LDH and other cytotoxic substances by necrotic cells into the medium. Thus, the existence of LDH in culture medium represents an indirect index of the membrane permeability of the treated cells. As shown in Fig. 2, 72 h of incubation with a chloroformic extract of *Celtis aetnensis* (Tornab.) Strobl (5 µg/ml) did not cause LDH release, while a statistically significant increase in LDH release was observed in the Caco2 cells treated with 50 and 250 µg/ml of extract (Fig. 2).

Caspase determination. Western blot analysis of caspase-3 is considered a good marker of apoptosis. As shown in Fig. 3, the treatment of Caco2 cells with a chloroformic extract of *Celtis aetnensis* (Tornab) Strobl (5-50-250 µg/ml) induced apoptotic cell death. This effect was also evident at the lowest dosage (5 µg/ml).

ROS assay. ROS are believed to be involved in cell death induced by a variety of stimuli and various antitumoral agents. In order to test the hypothesis that chloroformic extract-induced cell death may be mediated by an elevation in ROS levels, a fluorescent probe, DCFH-DA was used for ROS determination. This probe diffuses into the cells, intracellular esterases hydrolyze the acetate groups and the resulting DCFH then reacts with intracellular oxidants resulting in the observed fluorescence.

The intensity of fluorescence (FI) is proportional to the levels of intracellular oxidant species. As shown in Fig. 4, the addition of the chloroformic extract of *Celtis aetnensis* (Tornab.) Strobl at 5, 50 and 250 µg/ml for 72 h caused a significant increase in FI with respect to the untreated Caco2 cells.
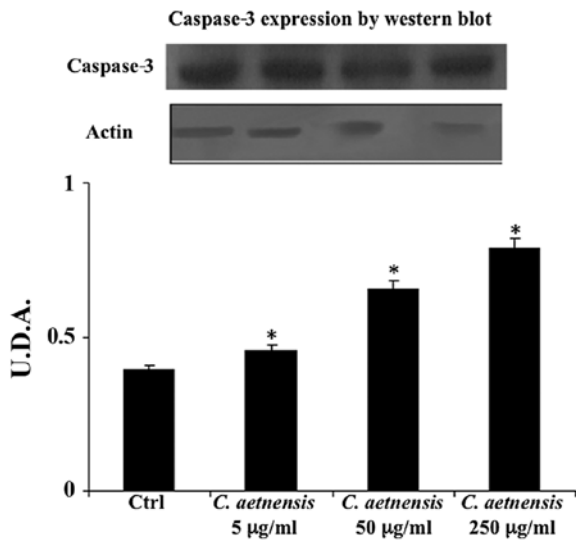
Figure 3. Immunoblotting of caspase-3 levels in the Caco2 cells untreated and treated for 72 h with a chloroformic extract of *Celtis aetnensis* (Tornab.) Strobl at different concentrations (5-250 µg/ml). Values are expressed as densitometric units corresponding to signal intensity present on autoradiographs. Values are the mean ± SD of four experiments performed in triplicate. Significant vs. untreated control cells, *P<0.001.
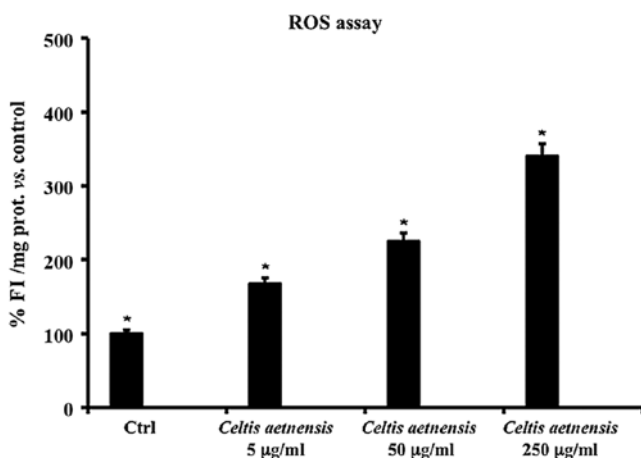

Figure 4. Intracellular oxidants in the Caco2 cells untreated and treated for 72 h with a chloroformic extract of *Celtis aetnensis* (Tornab.) Strobl at different concentrations (5-250 µg/ml). Values are the mean ± SD of four experiments in triplicate. Significant vs. untreated control cells, *P<0.001.
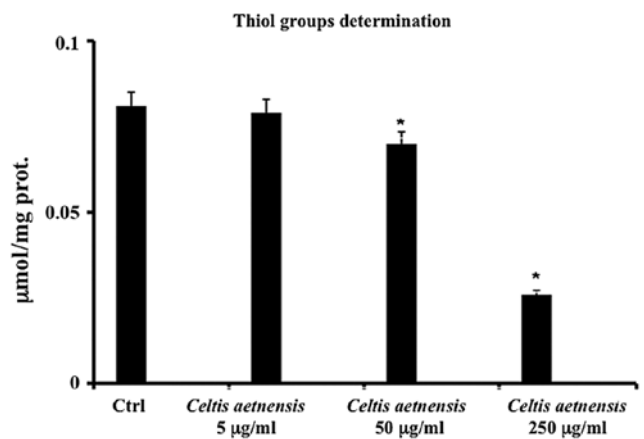

Figure 5. Thiol groups in the Caco2 cells untreated and treated for 72 h with a chloroformic extract of *Celtis aetnensis* (Tornab.) Strobl at different concentrations (5-250 µg/ml). Thiol groups are expressed as µmol/mg protein. Values are the mean ± SD of four experiments in triplicate. Significant vs. untreated control cells, *P<0.001.
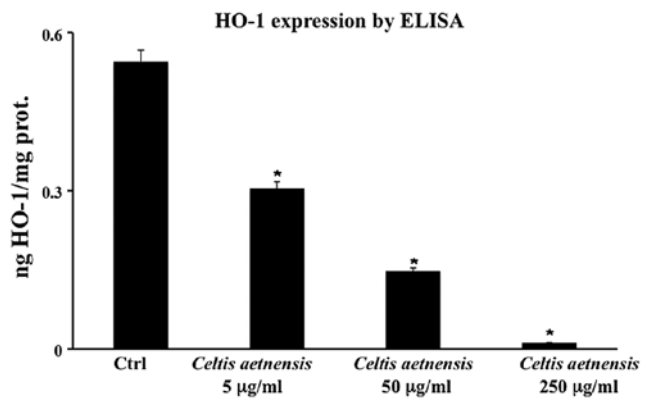

Figure 6. HO-1 levels in the Caco2 cells untreated and treated for 72 h with a chloroformic extract of *Celtis aetnensis* (Tornab.) Strobl at different concentrations (5-250 µg/ml). Results are expressed as ng/mg proteins. Values are the mean ± SD of four experiments in triplicate. Significant vs. untreated control cells, *P<0.001

Thiol groups. In order to further confirm the involvement of radical/oxidative species in the action mechanism of chloroformic extract of *Celtis aetnensis* (Tornab.) Strobl, thiol group levels (RSH) were measured in the Caco2 cells. The treatment of these cells with 50-250 µg/ml of the chloroformic extract of *Celtis aetnensis* (Tornab.) Strobl resulted in a significant reduction in the levels of RSH (Fig. 5); the lowest concentration of the chloroformic extract of *Celtis aetnensis* (Tornab.) Strobl did not alter the levels of thiol groups RSH with respect to the control.

HO-1 by ELISA. Fig. 6 shows that the addition of 5, 50 and 250 µg/ml of the extract to Caco2 cells, resulted in a significant decrease in HO-1 protein expression which was undetectable at the highest concentration of the extract (Fig. 6).

γ-GCS determination. No significant change in γ-GCS expression was observed in the Caco2 cells treated with the extract of *Celtis aetnensis* (Tornab.) Strobl with respect to the untreated cells (Fig. 7).

Discussion

Cancer, a malignant tumor or a neoplasm, is a generic term for a broad group of diseases that can affect any part of the body via failure of regulation of cell mitosis. The processes of cancer development are i) rapid and abnormal cell division and growth, ii) formation of malignant tumors, iii) invasion to nearby adjoining parts of the body, and iv) spread to other organs through the lymphatic system and/or bloodstream. Cancer remains one of the most threatening diseases worldwide affecting human health and quality of life in spite of
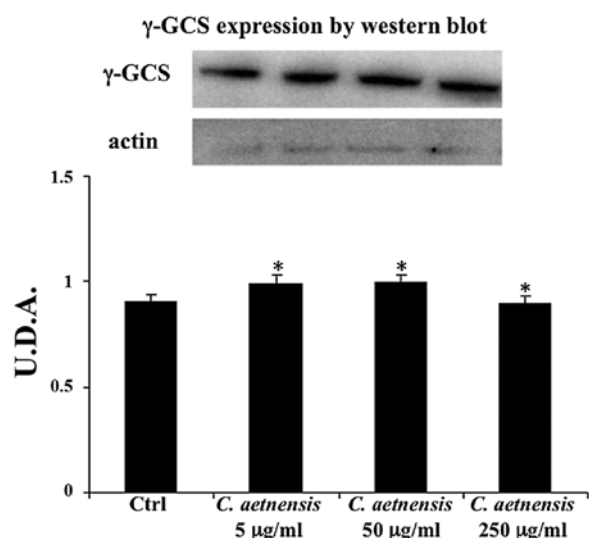
γ-GCS expression by western blot

Figure 7. Immunoblotting of γ-GCS levels in the Caco2 cells untreated and treated for 72 h with a chloroformic extract of *Celtis aetnensis* (Tornab.) Strobl at different concentrations (5-250 µg/ml). Values are expressed as densitometric units corresponding to signal intensity present on autoradiographs. Values are the mean ± SD of four experiments performed in triplicate. Significant vs. untreated control cells, *P<0.001.

the many recent advances in the knowledge of its molecular biology of induction and progression.

Cancer cells differ from normal cells due to the following properties: unlimited replication potential, absence of apoptosis, absence of telomere shortening, angiogenesis and metastasis. Colorectal carcinogenesis is related to the progressive loss of homeostatic control of cell proliferation, differentiation and apoptosis (26,27). The human body exerts protective effects against tumor development mainly through apoptosis, cell cycle arrest and immune responses. Apoptosis, also known as programmed cell death, involves cell blebbing, shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation. It can be triggered by the activation of tumor-suppressor genes, caspases, apoptosis-inducing factors, cytotoxic T cells and natural killer (NK) cells via a Fas ligand- or perforin/granzyme B-dependent pathway (28,29).

Natural compounds have been shown to affect molecular events involved in the initiation, promotion and progression of cancer, thereby inhibiting carcinogenesis. Furthermore, their inhibitory activity may ultimately suppress the final steps of carcinogenesis as well angiogenesis and metastasis.

Previous studies on ethnomedicine, together with extensive laboratory findings, indicate that flavonoids and triterpenic compounds play important roles in the prevention and treatment of cancer (10,30-33). Because of their safety, low toxicity and general acceptance as dietary supplements, fruits, vegetables, and other dietary elements (phytochemicals and minerals) are being investigated for the prevention of cancer. Extensive research over the past several decades has identified numerous dietary and botanical natural compounds with chemopreventive potential.

A number of reports have highlighted the important role of pro-oxidants and/or antioxidants by natural and synthetic

compounds in chemo-prevention for many cancers (34,35). Antioxidants have been thought to mainly suppress carcinogenesis during the initiation phase, since most act as radical scavengers, or inducers or inhibitors of xenobiotic metabolizing enzymes including phase I and II enzymes (36). In addition, some radical scavengers may also have pro-oxidative potential because of their conversion to more reactive or stable radicals after reaction with ROS.

There is a growing interest in flavonoids and triterpene esters and their synthetic derivatives due to their possible applications in cancer chemotherapy as anticancer and anti-inflammatory agents (37). Recently, it has been reported that a chloroformic extract of *C. philippinensis* twigs exhibited cytotoxic activity against the KB (human oral epidermoid carcinoma) cell line (10).

According to these findings, the results obtained in the present study showed that a chloroformic extract of *Celtis aetnensis* (Tornab.) Strobl significantly reduced the cell viability of Caco-2 cells and induced apoptotic and/or necrotic cell death in a concentration-dependent manner (Fig. 1). In particular, a chloroformic extract of *Celtis aetnensis* (Tornab.) Strobl induced apoptosis at the lowest concentration (5 µM), while higher dosages (50-250 µg/ml) were able to increase LDH release, a marker of necrotic death (Figs. 2 and 3). Here, the reported data regarding the MTT assay, LDH release and caspase expression suggest that the chloroformic extract of *Celtis aetnensis* (Tornab.) Strobl penetrated the cancer cells with consequent apoptosis and or necrosis depending on the concentration.

The involvement of ROS in necrotic/apoptotic cell death induced by different agents, such as oxidants, toxicants or drugs, has been suggested (38). It is interesting to note that many human cancer cell types exist in a highly oxidative state due to decreased antioxidant protective enzyme levels compared to their normal tissue counterparts. Thus, cancer cells may be more sensitive to ROS generation within the cells. Measurement of the cellular content of ROS after exposure to different concentrations of chloroformic extract of *Celtis aetnensis* (Tornab.) Strobl demostrated a significant dose-dependent increase in the levels of ROS (Fig. 4).

These results suggest that in the Caco-2 tumor cell line the chloroformic extract of *Celtis aetnensis* (Tornab.) Strobl acted as a pro-oxidant rather than as an antioxidant. These observations indicate that this extract may act indirectly and that its action may be mediated by other intracellular factors, likely targets of ROS.

These results are in agreement with other authors who demonstrated that phenolic compounds with high reducing ability may also act as pro-oxidants, thus generating ROS (39). The pro-oxidant activity of chloroformic extract of *Celtis aetnensis* (Tornab.) Strobl in Caco-2 cells was confirmed by results regarding thiol group determination; these endogenous antioxidants act concurrently by scavenging and/or reducing free radicals, breaking the peroxidative chain and thus allowing the repair of oxidatively damaged molecules (Fig. 5). The present study demonstrated that exposure of Caco2 cells to a chloroformic extract of *Celtis aetnensis* (Tornab.) Strobl induced a significant dose-dependent decrease in RSH levels suggesting that the antioxidant system was not able to buffer the overproduction of ROS (Fig. 5). The intracellular decrease

in RSH content appears to be a central event in Caco2 cell death induced by the chloroformic extract of *Celtis aetnensis* (Tornab.) Strobl. suggesting an interference of flavonoid and triterpenic compounds on the oxidant/antioxidant cell balance with consequent cell damage.

This study also evaluated the expression of HO-1, one of the most effective mechanisms for cellular protection against oxidative stress. The expression of HO-1 is increased by several types of cell stress including redox signals, heme, UV radiation, heavy metals, cytokines and ROS (40,41). The role of HO-1 in cancer biology is far from completely understood. In cancer, HO-1 has been described as a pro-tumoral molecule due to its anti-apoptotic effects on colon cancer and hepatoma in murine models and its proangiogenic effects in human pancreatic cancer (42,43). By contrast, in human tongue cancer, low HO-1 expression has been associated with an increased risk of developing lymph node metastasis (44).

In this study, we observed that the extract of *Celtis aetnensis* (Tornab.) Strobl decreased the expression of HO-1 (Fig. 6). Since HO-1 expression represents an important protective endogenous mechanism, its reduced expression, together with low RSH levels and high ROS production, may contribute to cell death.

To better understand the actions of the *Celtis aetnensis* (Tornab.) Strobl extract, we also evaluated γ-GCS expression, the rate-limiting enzyme in gluthatione synthesis, which can be considered as one of the major antioxidant enzymes.

A shown in Fig. 7 the expression of γ-GCS was not modified in the *Celtis aetnensis*-treated Caco-2 cells suggesting that lowered GSH levels, observed following extract treatment may be due to increase ROS generation rather than to inhibition of glutathione synthesis. Thus, the chloroformic extract of *Celtis aetnensis* (Tornab.) Strobl, inducing a decrease in antioxidant defenses, acts on Caco2 cells which are susceptible to oxidative damage, as a potentially powerful promoter of oxidative processes.

It is reasonable to hypothesize that some of the compounds present in *Celtis aetnensis* (Tornab.) Strobl extract, which have been shown to afford considerable protection against cancer by inhibiting, for example, oxidative stress, may also exhibit anticancer effects. Thus, our study supports the growing body of data suggesting the bioactivities of *Celtis aetnensis* (Tornab.) Strobl and its potential impact on cancer therapy and on human health. Identification of natural chemopreventive compounds is urgently needed to help the further design and administration of preclinical and clinical trials.

Acknowledgements

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