Research Article

Mediterranean diet and cardioprotection: Wild artichoke inhibits metalloproteinase 9

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Metalloproteinases (MMPs) are zinc-dependent endopeptidases responsible for the hydrolysis of various component of extracellular matrix such as gelatin and collagen. MMPs, namely MMP-2 and MMP-9 correlate with cardiovascular events in patients. We sought to determine whether supplementation with polyphenol-rich *Cynara cardunculus* (wild artichoke, traditional component of the Mediterranean diet) modulates MMP-9 expression and activity in cell cultures. A fully characterized *C. cardunculus* extract was able to inhibit, in a dose-dependent manner, the gelatinolytic activity of secreted MMP-9 and both secretion and human MMP-9 promoter-driven transcription. Analysis by HPLC of the *Cynara* extract identified polyphenols such as luteolin, apigenin, and caffeic acid, among others. However, testing a mix of the individual components suggested that the inhibitory effects of *C. cardunculus* are due to minor constituent fraction(s) as a whole. In promoting the health benefits of the Mediterranean diet, the role of wild plants as important meal components deserves further reappraisal.

Keywords: Antioxidants / Cardiovascular disease / Gelatinases / Mediterranean diet / Polyphenols

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1 Introduction

Knowledge about the precise role played on longevity and health by various micronutrients abundant in the Mediterranean diet is expanding [1, 2]. As an example, polyphenols have been shown to slow progression of degenerative diseases largely by modulating some enzymatic activities and intracellular signalling pathways [3, 4].

Metalloproteinases (MMPs) are zinc-dependent endopeptidases which are key mediators of the normal extracellular matrix (ECM) remodeling that takes place during development, tissue morphogenesis, and repair, and are responsible for the hydrolysis of various component of ECM such as gelatin and collagen [5]. In particular, MMPs regulate signals elicited by matrix molecules and have additional roles in the reorganization of tissues during pathological conditions such as inflammation, wound healing, atherosclerosis, and metastasis of cancer cells [6]. Among the different MMPs, gelatinases (gelatinase A, MMP-2; gelatinase B, MMP-9) have been shown to play key roles in atherosclerotic plaque growth and stability and in cancer [7]. Indeed, clinical and genetic studies correlated MMP-2 and MMP-9 with cardiovascular events in patients [8, 9]. Accumulating laboratory and clinical evidence indicate that therapeutic intervention may prevent acute coronary events in part by reducing inflammation including the expression and activity of MMPs [10]. Recent data suggest that antioxidant treatment inhibits gelatinolytic activity [11, 12]. In addition to drug treatment, dietary constituents, such as polyphenols, have been shown to be able to affect gelatinases activity [7]. Polyphenols are abundant components/micronutrients of the human diet, being important ingredients of fruits, vegetables, and beverages such as tea and wine. Because of their high abundance in the diet, especially in the Mediterranean diet, and the increased evidence about their antioxidant properties [4], much effort has been recently devoted to better elucidate the role of the dietary polyphenols in the prevention of diseases, mainly associated with oxidative stress.

The aim of the Local Food-Nutraceuticals project was to study the potential role of locally consumed food plant on

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Abbreviations: CHO, Chinese hamster ovary; **TPA,** phorbol esters

the incidence of cardiovascular disorders [2]. In fact, several pharmacological activities have been attributed to phenol-rich plants traditionally eaten in the Mediterranean area [2]. For example, *Cynara cardunculus* (wild artichoke) is being studied for its health-protective potential, in particular for hepatoprotective, anticancerogenic, and hypocholesterolemic properties [13] as well as for chemoprevention [14]. Recently, we reported on the hemodynamic effect of a well-characterized *C. cardunculus* extract, namely an enhanced endothelium-dependent vasorelaxation *in vitro* and *ex vivo* consequent to enhanced activity of nitric oxide (NO) synthase and prostacyclin production [15], as anticipated by *in vitro* data [16]. In this study, we sought to determine whether supplementation with *C. cardunculus* or its main identified constituents, namely luteolin-7-glycoside, apigenin, and caffeic acid modulates MMP-9 expression and activity in cell cultures. We also investigated whether the effects on MMP-9 activity were due to an individual *C. cardunculus* component or to the whole array of phenolic compounds present in the plant [17].

2 Materials and methods

Wild artichoke was collected and extracted as previously described [2]. Total polyphenolic content of the extract was determined by the Folin–Ciocalteau method, using gallic acid as the reference compound [18]. The extract was analyzed by HPLC-MS and its composition has been previously reported in details [16]. Based on the composition of the extract [16], we formulated a mixture containing the major components of *C. cardunculus* (luteolin-7-glycoside, apigenin, and caffeic acid (Sigma–Aldrich, Milan, Italy)). Namely, luteolin-7-glycoside was employed at a concentration of 86 μM, apigenin was 122 μM, and caffeic acid was 944 μM.

2.1 Cell culture

Chinese hamster ovary (CHO) cells were maintained in medium containing 10% FCS, at 37°C in a humidified atmosphere of 5% CO_2. CHO cells were used because they are easily transfectable, unlike macrophage cell lines, and secrete measurable amounts of endogenous MMP-9 whose activity is modulated by pharmacological treatment, as assessed by gelatin zymography [12, 19].

Cells were incubated for 24 h at 37°C in serum-free medium containing 0.2% BSA (Sigma–Aldrich) and increasing concentrations of *C. cardunculus* or vehicle (ethanol), in the presence or absence of phorbol esters (TPA, 50 ng/mL, Sigma–Aldrich) to stimulate MMP-9 expression [20]. At the end of treatment period, the gelatinolytic activity in the media was measured by gelatin zymography and cell protein content was estimated by the Bradford method [21].

2.2 Cytotoxicity assay

Cytotoxicity was evaluated by an *ad-hoc* colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, after removal of the medium and washing with PBS [16]. Each experiment was performed in duplicate wells and all experiments were performed at least three times.

2.3 SDS-PAGE zymography

To evaluate the effects of the extract on the activity of secreted MMP-9, aliquots of gelatinase-containing medium conditioned by CHO were assayed by gelatin zymography, on the basis of their molecular weights, under nonreducing conditions.

Briefly, samples underwent electrophoresis on 7,5% polyacrylamide gels containing 10% SDS and gelatin (1 mg/mL) as described [22]. The gels were then washed in 2.5% Triton X-100 (Sigma–Aldrich) at room temperature, then cut in strips and incubated overnight at 37°C in activation buffer (Tris 50 mM, pH 7.5, containing NaCl 150 mM, $CaCl_2$ 10 mM, and $ZnCl_2$ 1 μM) containing the tested compounds. Then, gels were stained with comassie brilliant blue R-250 (Sigma–Aldrich).

2.4 Determination of intracellular MMP-9

Intracellular MMPs activity was measured by gelatin zymography in CHO cell homogenates prepared in STEN buffer (Tris 1 M pH 7.6, NaCl 5 M, and 1% Nonidet P40). Cell protein solubilization was carried out for 20 min at 4°C, followed by centrifugation at 600 × *g* for 10 min. Total cellular protein was estimated by the Bradford method [21].

2.5 Transient transfection assays

CHO cells were transfected using the calcium phosphate coprecipitation technique [22]. Cells were plated at a density of 6×10^4 cells/well in DMEM containing 10% FCS, for 3 days at 37°C. A unique coprecipitate containing each reporter plasmid/luciferase plus pCMVb-gal was prepared and aliquoted in different wells to assure that all samples were transfected with the same amount of plasmid DNA (1.2 μg of luciferase plasmid + 0.3 μg of β-galactosidase plasmid DNA/well). After 16 h at 37°C, cells were washed with PBS and incubated for 24 h in medium containing the compounds to be tested or the vehicle (control).

2.6 Enzyme assay

Luciferase and β-galactosidase assays were performed using a luminometer (Lumat 9501, Berthold, Germany) and a microtiter plate reader (BioRad, Hercules, CA), respectively as previously described [23]. Luciferase activities

were normalized *versus* galactosidase activities. Results are expressed as the inhibition of normalized luciferase activities *versus* control of triplicate samples.

2.7 Statistical analysis

Each experiment was performed at least three times with different preparations of cells.

Gelatinase activity was quantified by scanning densitometry. We used a video camera and a computer analysis package (NIH Image 1.52 image analysis software) to perform a densitometric scanning for quantization of zymograms.

Data are presented as the means ± SD. Statistical analysis was done by two-tailed Student's *t*-test. A $p < 0.05$ was considered as statistically significant.

3 Results

To examine the effect of *C. cardunculus* on MMP-9 gelatinolytic capacity, we incubated CHO cells for 24 h with *C. cardunculus* extract (10^{-6}–10^{-9} M). Then, culture media were collected and analyzed by gelatin zymography. The addition of *C. cardunculus* dose-dependently reduced MMP-9 activity in confluent CHO cells up to 60% (Fig. 1, panel A, $p < 0.05$).

We and others have previously shown that the gelatinolytic capacity of MMP-9 is directly proportional to the amount secreted *in vitro* [20]. Therefore, to ascertain how

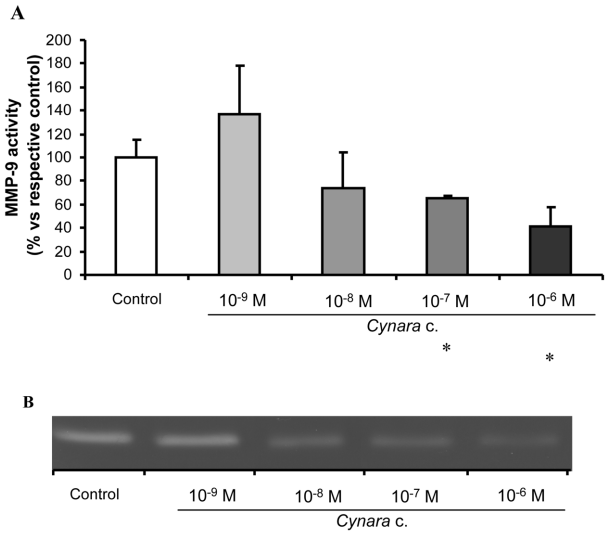

Figure 1. *C. cardunculus* inhibits MMP-9 activity in CHO cells (panel A). Cells were incubated for 24 h with increase in concentrations of the extract. Conditioned media were collected and MMP-9 activity was measured by gelatin zymography. Data are mean ± SD of three experiments performed in triplicate. $^{*}p < 0.05$ as compared with untreated cells. Panel B: representative gelatin zymograms showing the effect of the extract on MMP-9 gelatinolytic capacity.

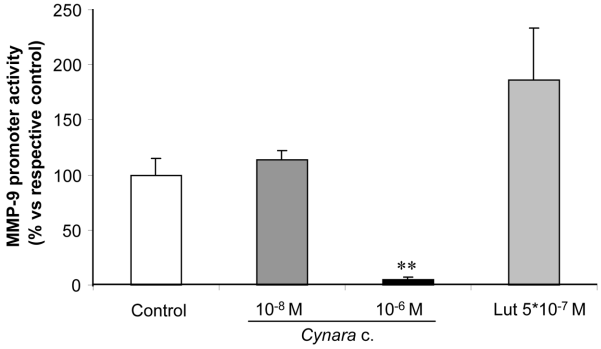

Figure 2. Effect of *C. cardunculus* or luteolin (lut) on MMP-9 promoter-driven transcription. CHO cells were transfected and incubated for 24 h with the indicated concentrations of compounds. A luciferase assay was then performed as described in Section 2. Luciferase activities were normalized to β-galattosidase activities. Values are expressed relative to controls. $^{*}p < 0.05$, $^{**}p < 0.01$.

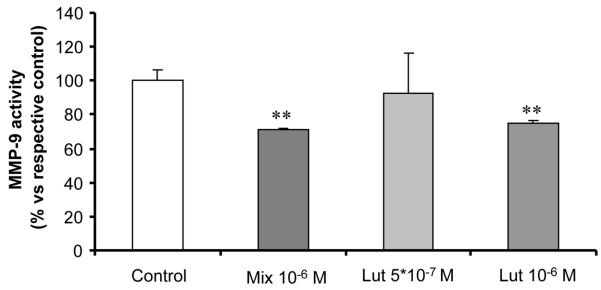

Figure 3. Effect of luteolin (lut) or of an extract-like mixture (Mix), containing the major identified constituents of the *C. cardunculus* extract (luteolin-7-glycoside, apigenin, and caffeic acid), on MMP-9 activity in CHO cell. Data were quantified by densitometry scanning and are expressed as the mean ± SD $^{**}p < 0.001$.

C. cardunculus could interfere with MMP-9 gelatinolytic potential, we investigated its effect on human MMP-9 promoter-driven transcription in CHO cells that were transiently transfected with a luciferase reported gene linked to the human MMP-9 promoter. As shown in Fig. 2 human MMP-9 promoter-driven transcription was significantly inhibited in cells treated with *C. cardunculus* as compared with untreated cells.

To exclude possible drug toxicity as the reason of the observed inhibitory effect, the MTT assay was employed to test for cellular toxicity. *C. cardunculus* extract did not affect cell viability at any of the concentrations tested (from 10^{-6}–10^{-9} M; data not shown).

Next we tried to identify the active compound(s) responsible for the modulation of MMP-9 expression. Supplementation of cells with a combination of the extract's major constituents (at a concentration of 10^{-6} M) reduced MMP-9 activity although less than what we observed with the original extract (Fig. 3). However, when we tested luteolin (one of the major component of the mixture) at a concentration

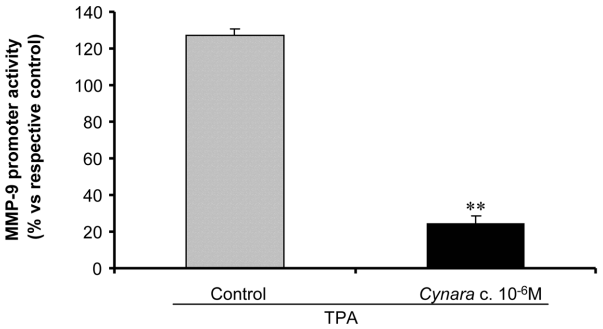

Figure 4. Effect of *C. cardunculus* on MMP-9 promoter-driven transcription. CHO cells were transfected and incubated for 24 h with TPA (50 ng/mL) and the indicated concentrations of compounds. A luciferase assay was then performed as described in Section 2. Luciferase activities were normalized to β-galattosidase activities. Values are expressed relative to control + TPA. $^{**}p < 0.01$.

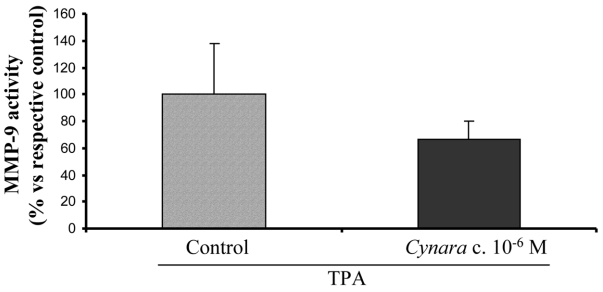

Figure 5. Effect of *C. cardunculus* on TPA-induced MMP-9 secretion by CHO cells. The cells were treated with *C. cardunculus* in the presence of TPA (50 ng/mL). Conditioned medium was collected after 24 h and MMP-9 activity evaluated by gelatin zymography.

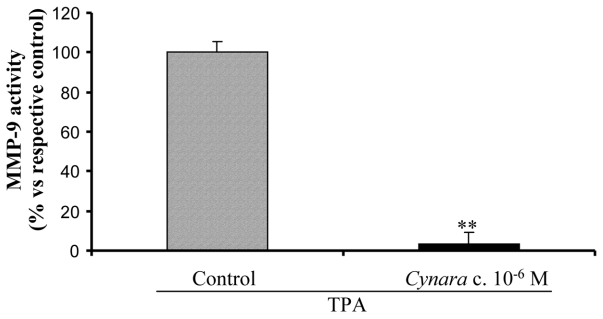

Figure 6. Effect of *C. cardunculus* on intracellular MMP-9 gelatinolytic activity. CHO were incubated for 24 h with *C cardunculus* in the presence of TPA (50 ng/mL). Intracellular MMP-9 activity was assessed by zymography in CHO cell homogenates prepared as described in Section 2. Data are expressed as the mean ± SD. $^{**}p < 0.001$.

identical to the one present in the extract (5×10^{-7} M) this did not modify either the promoter-driven transcription (Fig. 2) or subsequent secretion of MMP-9 (Fig. 3). These data indicate that luteolin *per se* is not responsible for the inhibition of MMP-9 activity.

To approximate pathological conditions in which MMP-9 is overexpressed, we incubated the cells with phorbol esters (TPA) which potently induces the expression and secretion of MMP-9 [24]. *C. cardunculus* extract (10^{-6} M) significantly decreased, as compared with untreated cells, TPA-induced MMP-9 promoter-driven transcription (Fig. 4). This was paralleled by a reduction in MMP-9 gelatinolytic potential in the conditioned media (Fig. 5), and almost completely abolished intracellular MMP-9 activity (Fig. 6), thus confirming the inhibitory effect also in the presence of a stimulus such as TPA.

4 Discussion

In this study, we show that a well-characterized *C. cardunculus* phenolic extract inhibits the gelatinolytic activity of secreted MMP-9, an enzyme linked to degenerative diseases [25]. In particular, the *C. cardunculus* extract was able to inhibit, in a dose-dependent manner, both secretion and human MMP-9 promoter-driven transcription. The working hypothesis is that the inhibitory properties of C. *cardunculus* were due to its high antioxidant content. In fact, several *in vitro* and *in vivo* studies demonstrated a relationship between the generation or exposure of ROS and MMP induction [25–27]. In particular, it has been shown that ROS activate MMP-9 [11] and that antioxidants are able to prevent such activation [28]. Yet, accumulating evidence suggests that polyphenolic compounds exert their biological activities *via* modulation of cell signaling, rather than through a mere antioxidant action [3]. In turn, it is conceivable that polyphenols such as those of *C. cardunculus* modulate MMP-9 activity in many ways, including metal chelation (especially by ortho-diphenols such as luteolin), catalytic activation, and modulation at the pre- or post-transcriptional levels [7, 29].

Analysis by HPLC of the *Cynara* extract identified known antioxidant polyphenols such as luteolin, apigenin, and caffeic acid among others [16]. The absorption and metabolism of these compounds after wild artichoke intake has been recently elucidated [30]. To ascertain whether the inhibitory effects of *C. cardunculus* on MMP-9 were due to an individual phenol, we tested luteolin, a strong ortho-diphenolic antioxidant, in the same experimental conditions that were employed for the *C. cardunculus* extract. We found, in agreement with Sartor *et al*. [31], that luteolin is not a selective MMP-2 and MMP-9 inhibitor. This finding confirms the hypothesis that different chemical structures characterize different mechanisms of action [29], yet it does not entirely rule out redox-regulated mechanisms. To follow up on this finding, we tested a combination of the phenolic antioxidants we identified in the extract. Again, partial reproduction of the extract did not entirely explain or reproduce the effects seen with the raw extract. In turn, while biological activities are generally attributed to anti-

oxidant polyphenols recovered in extracts, it appears to be very difficult to ascertain which of the individual compounds is/are responsible for the observed effects on MMPs. Furthermore, the ethanol-based extraction we used also extracts nonphenolic compounds such as fatty acid, alkaloids, or vitamins [2], which, albeit to a minor extent, might exert inhibitory activities on MMPs. As an example, medium-chain fatty acids, such as oleic and stearic acids, protect against gelatinolytic degradation [32]. GC analyses of fatty acids in the *C. cardunculus* extract (data not shown) revealed the presence of oleic and stearic acids, but at much lower concentrations than those demonstrated to inhibit MMP-9 [32]. Furthermore, fatty acids bind the first fibronectin type-II module present in both MMP-2 and MMP-9 [32] and our data show that the *C. cardunculus* extract was effective in inhibiting MMP-9 but not MMP-2 (data not shown).

Our data add new insights into the ability of polyphenols to act at the gene level, by inhibiting gene transcription. In fact, a review of the literature supports the hypothesis that dietary polyphenols such as green tea catechins or resveratrol work through the inhibition of gelatinases' gene expression and secretion [7, 33, 34]. This molecular mechanism can be in part related to the molecular-driven transcription involved in MMP-9 gene modulation. *In vitro* studies report that quercetin and other antioxidants reduce the binding of NF-κB and AP-1 transcription factors to DNA [35]. *In vivo* data are often difficult to obtain and this applies also to the modulation of MMP-9 gene transcription.

In summary, our results show that *C. cardunculus*, a wild plant traditionally part of the Mediterranean diet, is a powerful inhibitor of MMP-9, by interfering with its transcription and production. The inhibitory effects most probably are not due to an individual component of *C. cardunculus*, but, rather, to minor constituent fraction(s) as a whole. In promoting the health benefits of the Mediterranean diet, the role of wild plants as important meal components deserves further reappraisal [36].

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